Yeast and Mammalian RNA Polymerase II Holoenzymes

by

David M. Chao

A.B./A.M. Biology
Harvard University, 1989

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

MAY 1997

Signature of Author:_____
Department of Biology
May 19, 1997

Certified and Accepted by:_____
Richard A. Young
Professor of Biology
Thesis Supervisor
Chairman, Committee for Graduate Students

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Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology at the Massachusetts Institute of Technology

ABSTRACT

Components and regulators of yeast and mammalian RNA polymerase II holoenzymes were isolated and characterized. First, subcomplexes and components of the yeast RNA polymerase II holoenzyme were isolated and identified. A complex containing SRB and SWI/SNF regulatory proteins was purified over an affinity chromatography column containing the carboxyl terminal repeat domain of RNA polymerase II. The SRB/SWI/SNF complex stimulates transcription in vitro and contains chromatin remodeling and CTD kinase activities. Additional components of the SRB/SWI/SNF complex were identified by mass spectrometry. Second, hSRB7, a human homolog of a yeast SRB gene, was identified as a component of a mammalian RNA polymerase II holoenzyme. Finally, a yeast homolog of the mammalian negative cofactor NC2 was purified and shown to repress transcription by the RNA polymerase II holoenzyme in vitro.

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

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Chapter 1: Introduction: Factors involved in transcription initiation

Overview

The regulation of gene expression is fundamental to living processes. In the simple eukaryote Saccharomyces cerevisiae, 4,665 genes give rise to a steady state level of approximately 15,000 mRNA transcripts (Velculescu, Zhang et al. 1997). The steady-state number of transcripts per gene per cell varies from 0.3 to 200 copies (Velculescu, Zhang et al. 1997). The steady state level of an average mRNA is 1 to 2 copies per cell (Struhl and Davis 1981). The regulation of transcription initiation is thought to account for much of the variation in transcript levels.

The RNA polymerase II holoenzyme is the apparatus that initiates the transcription of mRNA in vivo. The holoenzyme contains RNA polymerase II; the general transcription factors TFIIB, TFIIF and TFIIH; and regulatory proteins such as the SRB and SWI/SNF proteins. Additional general transcription factors and gene specific and general regulatory proteins also play a role in initiating transcription.

As background, I describe our knowledge of the factors involved in transcription initiation in Chapter 1. In Chapter 2, I describe the identification and isolation of a complex containing SRB regulatory proteins. In Chapter 3, I describe the identification of the SWI/SNF and other proteins as components of the SRB complex. In Chapter 4, I describe the isolation of a human SRB gene and its association with a mammalian RNA polymerase II holoenzyme. In Chapter 5, I describe the purification and characterization of the negative regulator NC2 from yeast. In Chapter 6, I discuss the RNA polymerase II holoenzyme in the context of models for preinitiation complex formation

and transcriptional activation and as an example of a large protein complex involved in processing regulatory signals.

1. Components of the RNA polymerase II holoenzyme

The best characterized form of yeast RNA polymerase II holoenzyme contains RNA polymerase II; the SRBs; the general transcription factors TFIIB, TFIIF and TFIIH; the SWI/SNF regulatory proteins; as well as GAL11, RGR1, SIN4 and ROX3 (reviewed in Berk 1995; Koleske and Young 1995; Halle and Meisterernst 1996; and Carey 1995). However, multiple forms of RNA polymerase II holoenzyme have been isolated from yeast and mammalian cells. All RNA polymerase II holoenzymes isolated so far contain RNA polymerase II, at least one SRB protein, and at least one general transcription factor. The subset of general transcription factors associated with the holoenzyme appears to vary in the various preparations. One explanation for these results is that there are different forms of holoenzyme in vivo. As is the case with the SWI/SNF complex (Wang, Xue et al. 1996) and TFIID (Dikstein, Zhou et al. 1996), there may be different forms of holoenzyme in different cell types or under different physiological conditions. The composition of the holoenzyme may also vary during the various steps in transcription initiation and elongation. Another explanation is that the various purified forms of holoenzyme represent artifactual subcomplexes of what exists in vivo. The dilution, hydrodynamic shearing and extreme ionic conditions of protein purification are likely to be extremely disruptive to complexes accustomed to the concentrated environment of the nucleus. In support of this notion, rapid immunoprecipitation procedures yield holoenzyme complexes containing more general transcription factors than

versions purified by conventional chromatography (Ossipow, Tassan et al. 1996; Chris Wilson, unpublished). It is possible that improved purification procedures will allow the isolation of more complete holoenzymes which will provide an improved picture of the transcriptional apparatus in vivo.

RNA polymerase II

RNA polymerase II is the enzyme responsible for catalyzing the production of mRNA from protein-encoding genes (Roeder and Rutter 1969). A structure of RNA polymerase II at 16 angstrom resolution is available (Darst, Edwards et al. 1991). Low resolution structures of RNA polymerase II complexed with TFIIB and TFIIE and lacking a CTD are also available (Leuther, Bushnell et al. 1996). These low resolution structures provide a gross view of RNA polymerase II but provide little insight into how the enzyme functions. With such low resolution, structural features cannot be assigned to individual residues and cannot be interpreted in light of the available genetic information.

The enzyme contains 12 subunits, ranging in size from 220 to 10 kD (reviewed in Young 1991). The subunit composition reveals common evolutionary origins and homologous functions among the three RNA polymerases. Some of the RNA polymerase II subunits are shared with RNA polymerases I and III, and some have homologous counterparts in the other polymerases. The composition of core RNA polymerase II varies with the physiological state of the cell. RPB4 and RPB7 form a subcomplex which can be dissociated from the other RNA polymerase II subunits (Edwards, Kane et al. 1991). Their addition to purified RNA polymerase II lacking these subunits stimulates transcription in vitro (Edwards, Kane et al. 1991). In vivo, their association with the other polymerase II subunits is increased by physiological

stress (Choder and Young 1993), an observation suggesting that the RPB4 and RPB7 subunits couple RNA polymerase II activity with broad physiological changes. Alterations in the subunit composition of RNA polymerase II may represent a means for coordinately regulating entire sets of genes.

An interesting feature of RNA polymerase II is a structure found at the C-terminus of its largest subunit. The carboxyl-terminal repeat domain (CTD) consists of multiple heptapeptide repeat with the sequence YSPTSPS. The sequence is conserved among eukaryotes but is absent from viral RNA polymerases and RNA polymerase I and III (reviewed in Chao and Young 1991 and Corden 1990). There are 26 or 27 repeats in the yeast CTD (Allison, Moyle et al. 1985) and 52 copies in the mouse version (Corden, Cadena et al. 1985; Allison, Wong et al. 1988). The length of the CTD increases roughly with the genomic complexity of an organism while the conservation of the individual repeats decreases. The evolutionary divergence of the CTD may reflect the increased regulatory demands of coordinating transcription in more complex genomes.

The CTD is required for the viability of yeast (Nonet, Sweetser et al. 1987; Allison, Wong et al. 1988), Drosophila (Zehring, Lee et al. 1988) and mammalian cells (Bartolomei, Halden et al. 1988). More specifically, the CTD is involved in responses to transcriptional activators in both yeast (Allison and Ingles 1989; Scafe, Chao et al. 1990) and mammalian cells (Gerber, Hagmann et al. 1995). In these in vivo studies, mutants containing truncated CTDs show a defect in the response to a subset of activators. Likewise, in vitro studies indicate that the CTD is involved in transcription from some promoters (Thompson, Steinberg et al. 1989; Liao, Taylor et al. 1991) but not others (Zehring, Lee et al. 1988; Buratowski and Sharp 1990; Zehring and Greenleaf 1990). These studies in vivo and in vitro indicate that different

promoters vary in their requirement for the CTD and provide support for the notion that the influence of different regulatory mechanisms varies from promoter to promoter.

Besides a role in transcription initiation, the CTD may play a role in linking transcription with splicing. Several SR-related proteins have been shown to interact with murine CTD in a two hybrid assay (Yuryev, Patturajan et al. 1996). Consistent with these results, mammalian RNA polymerase II containing a phosphorylated CTD associates with splicing factors in vitro (Mortillaro, Blencowe et al. 1996; Kim, Du et al. 1997). At the subcellular level, mammalian RNA polymerase II containing a phosphorylated CTD localizes to subnuclear speckles containing splicing factors (Bregman, Du et al. 1995; Mortillaro, Blencowe et al. 1996). Overexpression of the CTD alone appears to disrupt the subnuclear localization of splicing factors and blocks the accumulation of at least one spliced message (Du and Warren 1997). Besides splicing, the CTD may also play a role in processing of the 3' end and termination of transcription downstream of the poly(A) site of transcripts. Cleavage and polyadenylation factors bind specifically to the CTD in vitro, and, in mammalian cells, a mutant containing a truncated CTD is defective in these processes (McCracken, Fong et al. 1997). Extending these observations to the yeast system, where mutations in the CTD are more easily and directly studied, would be helpful in substantiating their physiological significance. However, the lack of yeast mutants that specifically disrupt CTD phosphorylation and the technical challenge of observing speckles in the small yeast nucleus represent significant obstacles.

A proportion of the RNA polymerase II molecules in cells are phosphorylated on the CTD in vivo (Cadena and Dahmus 1987; Kolodziej,

Woychik et al. 1990, reviewed in Dahmus 1996). There is some controversy as to which residues are phosphorylated in the CTD. RNA polymerase II CTDs isolated by different procedures contain phosphoserine and phosphothreonine in one case (Zhang and Corden 1991) and also phosphotyrosine in another (Baskaran, Dahmus et al. 1993). The structural consequence of CTD phosphorylation may be a conformational change. While a synthetic CTD peptide appears to be mostly disordered in solution (Cagas and Corden 1995), phosphorylation does lead to a significant increase in the CTD's apparent molecular weight by gel filtration (Zhang and Corden 1991).

The role of CTD phosphorylation in vivo has been difficult to establish. Phosphorylation of the CTD correlates with promoter clearance in vitro (Payne, Laybourn et al. 1989; Laybourn and Dahmus 1990). In Drosophila cells, the CTD becomes phosphorylated after RNA polymerase II switches from a paused to an elongating form (O'Brien, Hardin et al. 1994). Furthermore, in this system, the level of CTD phosphorylation varies from promoter to promoter (O'Brien, Hardin et al. 1994). It is important to note that these studies are correlative. They do not show that CTD phosphorylation is required for promoter clearance or elongation. In fact, in one in vitro system, phosphorylation of the CTD is not required for proper initiation, promoter clearance or transcription of the Adeno Major Late promoter (Serizawa 1993). In another in vitro system, CTD phosphorylation is required for transcription of the DHFR promoter (Akoulitchev, Makela et al. 1995). The requirement for CTD phosphorylation in crude in vitro transcription systems is likely to involve factors absent from highly purified systems. In the yeast system, transcription reactions reconstituted with crude fractions are sensitive to the addition of a kinase inhibitor while reactions reconstituted with highly

purified factors are not (Li and Kornberg 1994). These in vitro results are consistent with a model where a kinase phosphorylating the CTD overcomes inhibition by a negative regulator. Confirmation of the existence and identity of such a factor would provide useful insights into the physiological role of CTD phosphorylation. Phosphorylation of the CTD also appears to inhibit the binding of TBP to the CTD (Usheva, Maldonado et al. 1992), but there is not yet any genetic data that supports a role for this interaction in vivo.

Several kinases with the ability to phosphorylate the CTD in vitro have been identified. The yeast kinase CTK1 is the only kinase whose mutation appears to affect on CTD phosphorylation in vivo. CTK1 phosphorylates CTD in vitro (Lee and Greenleaf 1989) and has a very limited effect on CTD phosphorylation in vivo (Lee and Greenleaf 1991). Yeast and mammalian versions of the general transcription factor TFIIH both phosphorylate the CTD in vitro (Feaver, Gileadi et al. 1991; Lu, Zawel et al. 1992), but whether the CTD is a physiologically relevant substrate has yet to be shown. Other CTD kinases include a CDK (Cisek and Corden 1989) and a Drosophila elongation factor P-TEFb (Marshall and Price 1995; Marshall, Peng et al. 1996). In neither case has the CTD kinase activity been shown to be physiologically relevant.

Mutations in SRB10 and 11, a kinase-cyclin pair, suppress CTD truncation mutants. The CTD kinase activity of an RNA polymerase II holoenzyme containing a mutant form of SRB10 is 10 fold lower than that of a wild-type holoenzyme (Liao, Zhang et al. 1995). Mutations in SRB10 and 11 have not yet been shown to affect CTD phosphorylation in vivo.

Studying the role of CTD phosphorylation in vivo has proven to be challenging. One obstacle to genetic studies is difficulty in demonstrating that mutation of any of the putative CTD kinases actually affects CTD

phosphorylation in vivo. One reason may be the presence of redundant kinases, whose activity would make it difficult to observe effects when only one kinase is mutated. Even if a mutation affecting CTD phosphorylation is isolated, it may be difficult to distinguish between direct and indirect effects. Given that CTD phosphorylation correlates with an elongating polymerase, it is likely that any mutation blocking a step before elongation would indirectly affect the steady state level of CTD phosphorylation.

SRB proteins

Mutants containing a truncated CTD are cold-sensitive, temperature-sensitive (Nonet, Sweetser et al. 1987), unable to grow without inositol (Nonet and Young 1989), and unable to use pyruvate as a carbon source (Thompson, Koleske et al. 1993). The SRB (Suppressors of RNA polymerase II or B) genes are allele-specific extragenic suppressors of a CTD-truncation mutant (Nonet and Young 1989; Koleske, Buratowski et al. 1992; Thompson, Koleske et al. 1993; Hengartner, Thompson et al. 1995; Liao, Zhang et al. 1995). This genetic interaction between the SRBs and the CTD is consistent with the biochemical observation that the SRBs bind to a CTD affinity column (Thompson, Koleske et al. 1993). An interaction between the SRBs and the CTD is also supported by the observation that SRBs are dissociated from RNA polymerase II by treatment with a monoclonal antibody directed against the CTD (Kim, Bjorklund et al. 1994).

The SRBs are a hallmark of the RNA polymerase II holoenzyme. The holoenzyme contains approximately 20% of RNA polymerase II molecules and 100% of the SRBs in the cell (Koleske and Young 1994). The RNA polymerase II holoenzyme is responsive to the activator GAL4-VP16 in vitro (Kim, Bjorklund et al. 1994; Koleske and Young 1994). A subcomplex

containing SRB proteins allows a reconstituted system containing purified general transcription factors to respond to activators (Kim, Bjorklund et al. 1994; Hengartner, Thompson et al. 1995). Consistent with these functional results, the RNA polymerase II holoenzyme and the SRB subcomplex can bind to an activator in vitro (Hengartner, Thompson et al. 1995)

A shift to the non-permissive temperature leads to a rapid cessation of mRNA synthesis in a cell containing temperature sensitive mutations in SRB4 (Thompson and Young 1995). SRB4 activity is required for transcription from 10 of 10 promoters examined. Temperature sensitive mutations in SRB6 show the same effect (Thompson and Young 1995). These results indicate that the holoenzyme is the form of RNA polymerase II required for class II transcription in yeast cells because all of the detectable SRB4 and SRB6 are associated with the holoenzyme.

SRBs also show genetic interactions with additional genes. Mutations in SRB8 and SRB9 (Song, Treich et al. 1996) and in SRB10 and SRB11 (Kuchin, Yeghiayan et al. 1995) suppress mutations in SNF1, a gene involved in regulation of the SUC2 gene. Mutations in SRB10 also suppress a mutation in the α2 gene, a gene-specific repressor (Wahi and Johnson 1995), and give rise to defects in the levels of meiosis-specific transcripts (Surosky, Strich et al. 1994). SRB8, SRB9, SRB10 and SRB11 are similar in that their deletion phenotype is slow-growth and flocculence and in that loss of function mutations suppress the CTD truncation mutants (Hengartner, Thompson et al. 1995; Liao, Zhang et al. 1995).

SRB10 and SRB11 encode a kinase/cyclin pair (Liao, Zhang et al. 1995). Loss of function mutations in SRB10 can either increase or decrease expression levels depending upon the promoter. Loss of SRB10 function leads to increased transcription from the SUC2 promoter (Kuchin, Yeghiayan et al.

1995) and from the promoters of a-specific genes (Wahi and Johnson 1995). In contrast, at the GAL1-10 promoter, loss of SRB10 function leads to a decrease in activated transcription (Liao, Zhang et al. 1995). It is not yet clear whether SRB10 and SRB11 play a direct role in mediating transcriptional repression or whether SRB10 and SRB11 play an indirect role by affecting the levels of factors involved in repression. It has been difficult to reproduce the effects of SRB10 mutation on transcription in vivo with an in vitro system. In a system reconstituted with purified RNA polymerase II holoenzyme, a mutation inactivating SRB10 has no effect on either basal or GAL4-VP16 activated transcription. However, mutating SRB10 does decrease the holoenzyme's CTD kinase activity (Liao, Zhang et al. 1995). It remains to be seen whether the failure to observe transcriptional defects of SRB10 mutations in vitro results from the absence of negative regulators, the use of an exceptionally powerful viral activator, or some other factor.

A mammalian SRB homologue of SRB7 has been identified and used to isolate a mammalian RNA polymerase II holoenzyme (Chao, Gadbois et al. 1996). Human SRB7 is present in two other preparations of holoenzyme (Ossipow, Tassan et al. 1995; Maldonado, Shiekhattar et al. 1996; Cho, Maldonado et al. 1997). CDK8 and cyclin C associate with RNA polymerase II and have been proposed to be homologues of SRB10 and 11 respectively (Tassan, Jaquenoud et al. 1995; Leclerc, Tassan et al. 1996). The tight sequence conservation among members of the CDK and cyclin families makes it difficult to establish whether CDK8 and cyclin C are actually functional homologues of SRB10 and 11. There is, however, some circumstantial evidence for a relationship. hSRB7, CDK8 and cyclin C have been reported to cofractionate as a 1.5 MD complex in gel filtration experiments (Gold, Tassan et al. 1996).

Some preparations of RNA polymerase II holoenzyme respond to the activator GAL4-VP16 (Maldonado, Shiekhattar et al. 1996). Other preparations fail to respond to activators in the absence of additional coactivators (Ossipow, Tassan et al. 1995; Chao, Gadbois et al. 1996). The various forms of mammalian RNA polymerase II are too poorly characterized to attribute these differences to the presence or absence of specific proteins.

TFIIB

TFIIB is present in some preparations of yeast holoenzyme but not others (Koleske, Chao et al. 1996). Yeast TFIIB has been cloned (Pinto, Ware et al. 1992) and purified (Tschochner, Sayre et al. 1992) and is a single subunit of 38 kD. It is extremely similar in sequence to human TFIIB (Ha, Lane et al. 1991). The crystal structure of a complex containing TFIIB bound to TBP and DNA (Nikolov, Chen et al. 1995) and the solution structure of a C-terminal fragment (Bagby, Kim et al. 1995) are both known.

TFIIB is required for cell viability (Pinto, Ware et al. 1992) and is involved in selection of the transcription start site. Mutations in TFIIB alter the position of the start site in vivo (Pinto, Ware et al. 1992; Pinto, Wu et al. 1994) Similarly, experiments swapping TFIIB and RNA polymerase II from different species indicate that TFIIB is involved in selecting the start site in vitro (Li, Flanagan et al. 1994). Affinity chromatography and surface-plasmon resonance experiments demonstrate an interaction between TFIIB and RNA polymerase II (Tschochner, Sayre et al. 1992; Bushnell, Bamdad et al. 1996) and a low resolution two dimensional crystal structure shows TFIIB bound to RNA polymerase II (Leuther, Bushnell et al. 1996). In vivo work also supports an interaction between TFIIB and RNA polymerase II. A mutation in the RNA polymerase II subunit RPB9 suppresses the start-site selection defect of a

TFIIB mutation (Sun, Tessmer et al. 1996). Furthermore, TFIIB mutants defective in start-site selection in vivo show diminished binding to RNA polymerase II in vitro (Bushnell, Bamdad et al. 1996).

Like many general transcription factors, TFIIB binds directly to a VP16 column (Lin, Ha et al. 1991). VP16 binding correlates with the ability of mutant TFIIB molecules to support activated transcription in vitro (Roberts, Ha et al. 1993). The interaction between VP16 and TFIIB increases the number of functional pre-initiation complexes by recruiting TFIIB to the promoter (Lin and Green 1991) and by causing a conformational change in TFIIB (Roberts and Green 1994).

TFIIF

TFIIF is a component of all reported forms of yeast RNA polymerase II holoenzymes (Kim, Bjorklund et al. 1994; Koleske and Young 1994; Koleske, Chao et al. 1996).

Mammalian TFIIF has two subunits with molecular weights of 30 and 74 kD (Flores, Ha et al. 1990). Yeast TFIIF has three subunits with molecular weights of 30, 50 and 105 kD (Henry, Sayre et al. 1992). The 50 kD and 105 kD subunits are homologous to the small and large subunits of the mammalian enzyme (Henry, Campbell et al. 1994). The 30 kD subunit is loosely associated with the other two subunits. It is stimulatory but not essential for transcriptional activity in vitro. The 30 kD subunit also associates with the TBP-TAF complex and with the SWI/SNF complex (Henry, Campbell et al. 1994). One explanation for the presence of the same 30 kD subunit in all three complexes is that the subunit performs an auxiliary function common to all three complexes. Another explanation is that TFIIF, TAFs and SWI-SNF are all part of a larger complex, such as the holoenzyme, and that the forms

observed in vitro represent different breakdown products of the larger complex.

Mammalian TFIIF contains RAP30 (Sopta, Burton et al. 1989) and RAP74 (Flores, Maldonado et al. 1988; Finkelstein, Kostrub et al. 1992), proteins associated with RNA polymerase II. TFIIF associates with RNA polymerase II in a variety of experimental contexts, including affinity chromatography (Sopta, Carthew et al. 1985), co-sedimentation (Flores, Maldonado et al. 1989; Henry, Sayre et al. 1992) and surface plasmon resonance (Bushnell, Bamdad et al. 1996). Rat TFIIF reduces the affinity of RNA polymerase II for free DNA, an activity similar to that of bacterial sigma factors (Conaway and Conaway 1990). Besides a role in initiation, TFIIF may play a role in elongation as it stimulates elongation by RNA polymerase II in vitro (Flores, Maldonado et al. 1989; Bengal, Flores et al. 1991).

The 50 and 105 kD subunits of yeast TFIIF are required for cell viability (Henry, Campbell et al. 1994). In addition, genetic results suggest a functional interaction between TFIIB and TFIIF. A mutation in the largest subunit of TFIIF suppresses defective start-site selection in a TFIIB mutant (Sun and Hampsey 1995).

In a two hybrid assay, the largest subunit of TFIIF also interacts with the transcriptional activator Serum Response Factor, (Joliot, Demma et al. 1995). Consistent with the two hybrid results, a mutation disrupting the interaction in vivo supports responds to Sp1 but not the Serum Response Factor in a reconstituted in vitro transcription system (Joliot, Demma et al. 1995).

TFIIH

TFIIH is found in some preparations of yeast RNA polymerase II holoenzyme (Koleske and Young 1994; Koleske, Chao et al. 1996) but not in others (Kim, Bjorklund et al. 1994).

Yeast TFIIH can be dissociated into three components: a five subunit core, TFIIK and the SSL2 protein (Svejstrup, Wang et al. 1995). Mammalian TFIIH is less well characterized than the yeast version but appears to have homologous counterparts for most if not all of the yeast subunits (reviewed in Svejstrup, Vichi et al. 1996).

Core TFIIH contains five subunits: RAD3, TFB1 and SSL1 and 50 and 38 kD subunits (Feaver, Svejstrup et al. 1993). The subunits of core TFIIH are shared with the form of TFIIH involved in nucleotide excision repair (Svejstrup, Wang et al. 1995). The role of TFIIH in DNA repair is reviewed in Seroz, Hwang et al. 1995; Chalut, Moncollin et al. 1994; Hoeijmakers, Egly et al. 1996; Svejstrup, Vichi et al. 1996.

The TFIIK subcomplex contains the kinase KIN28 (Feaver, Svejstrup et al. 1994) and its cyclin partner CCL1 (Svejstrup, Feaver et al. 1996). The mammalian counterparts of KIN28 are CDK7 and cyclin H respectively (Roy, Adamczewski et al. 1994; Serizawa, Makela et al. 1995; Shiekhattar, Mermelstein et al. 1995). The crystal structure of cyclin H is similar to that of other cyclins (Andersen, Poterszman et al. 1996; Kim, Chamberlin et al. 1996). Mammalian TFIIH has CDK-activating kinase (CAK) activity in vitro (Roy, Adamczewski et al. 1994; Serizawa, Makela et al. 1995; Shiekhattar, Mermelstein et al. 1995) while an immunopurified complex containing KIN28 and CCL1 does not (Cismowski, Laff et al. 1995). A temperature-sensitive mutation in KIN28 shows a transcriptional defect but no defect in the phosphorylation of CDC28, a target of CAK (Cismowski, Laff et al. 1995). In contrast, yeast CAK1/CIV1, whose product has CAK activity in vitro, shows a

cell-cycle defect and reduced CDC28 activity but does not exhibit a transcriptional defect (Espinoza, Farrell et al. 1996; Kaldis, Sutton et al. 1996; Thuret, Valay et al. 1996). With the exception of the in vitro phosphorylation data with CDK7-cyclin H, these results are consistent with roles for KIN28-CCL1 and CDK7-Cyclin H in transcription but not cell-cycle regulation. An alternative explanation including the in vitro CAK results is that CDK7-Cyclin H plays a role in regulating the cell cycle in mammals, a role that KIN28-CCL1 does not play in yeast. There is some precedent for homologous CDKs playing different roles in mammalian and yeast cells. Determining whether CDK7 and Cyclin H mutants have a cell-cycle arrest phenotype in mammalian cells would help clarify the issue.

Besides CDC2/CDC28, another target for the TFIIH kinase may be the CTD. Both yeast (Feaver, Gileadi et al. 1991) and mammalian (Lu, Zawel et al. 1992) TFIIH phosphorylate the CTD in vitro. More specifically, the TFIIK subcomplex has CTD kinase activity in vitro (Feaver, Svejstrup et al. 1994). The CTD kinase activity is stimulated by TFIIE in vitro (Lu, Zawel et al. 1992; Ohkuma and Roeder 1994). In addition to the CTD, mammalian TFIIH phosphorylates TBP, the large subunit of TFIIE and the large subunit of TFIIF in vitro (Ohkuma and Roeder 1994).

Basal and activated transcription are normal in a purified system containing a mutant TFIIH lacking CTD kinase activity (Makela, Parvin et al. 1995). Similarly, basal transcription is normal in a reconstituted system where CTD kinase activity is inhibited by the nucleotide analog H-8 (Serizawa, Conaway et al. 1993). However, in a cruder in vitro system, the CTD kinase activity of TFIIH is required for transcription from the DHFR promoter (Akoulitchev, Makela et al. 1995). TFIIH may be required to overcome a negative regulator contaminating crude in vitro transcription systems.

Support for this notion comes from work in an in vitro transcription system where TFIIH is not required. In this system, the addition of TFIIH overcomes inhibition by the non-histone chromosomal protein HMG2 (Stelzer, Goppelt et al. 1994).

Evidence also suggests that TFIIH plays a role in activation. Mammalian TFIIH binds to the activators VP16 and p53 on affinity columns (Xiao, Pearson et al. 1994). Certain activators stimulate transcriptional elongation(Yankulov, Blau et al. 1994; Blau, Xiao et al. 1996). The strength of these activators correlates with their affinity for TFIIH in vitro (Blau, Xiao et al. 1996). Similarly, the transcriptional regulator HIV Tat stimulates both elongation and phosphorylation of the CTD by TFIIH in vitro (Parada and Roeder 1996). A role for TFIIH in elongation is also suggested by the observation that microinjection of antibodies against TFIIH into Xenopus oocytes inhibits elongation on test promoters (Yankulov, Pandes et al. 1996).

SWI/SNF proteins

Yeast and human SWI/SNF proteins can be isolated as an eleven subunit complex (Cairns, Kim et al. 1994; Kwon, Imbalzano et al. 1994; Peterson, Dingwall et al. 1994) or associated with SRB proteins and the RNA polymerase II holoenzyme (Wilson, Chao et al. 1996). The composition of mammalian SWI/SNF complex appears to be different in different human cell types (Wang, Cote et al. 1996; Wang, Xue et al. 1996). SNF2/SWI2 appears to be a part of a large protein family whose members contain DNA-dependent ATPase activities (Laurent, Treich et al. 1993). Other members of the SNF2/SWI2 family have been identified as components of other chromatin remodeling activities such as NURF (Tsukiyama, Daniel et al. 1995) and RSC (Cairns, Lorch et al. 1996).

Purified SWI/SNF complex (Cote, Quinn et al. 1994; Imbalzano, Kwon et al. 1994; Kwon, Imbalzano et al. 1994), SRB/SWI/SNF complex and RNA polymerase II holoenzyme (Wilson, Chao et al. 1996) all exhibit ATP-dependent nucleosome disruption activity. SWI/SNF complex promotes the binding of TBP (Imbalzano, Kwon et al. 1994) and GAL4 (Cote, Quinn et al. 1994; Kwon, Imbalzano et al. 1994) to chromatin DNA templates. The disrupted nucleosome structure around DNA-bound Gal4 persists after removal of the SWI/SNF protein from the template (Owen-Hughes, Utley et al. 1996).

SWI/SNF genes influence the expression of the SUC2 and the HO genes (reviewed in Winston and Carlson 1992) and are required for normal transcription of at a large number of genes (Laurent, Treitel et al. 1990; Laurent and Carlson 1992; Peterson and Herskowitz 1992).

The nucleosome disruption activity of the SWI/SNF complex is consistent with the genetic interactions observed between the SWI/SNF genes and histone genes. Mutations in SNF2/SWI2, and SNF5, and SNF6 are suppressed by mutations in histones H2A and H2B (Hirschhorn, Brown et al. 1992), and mutations in SWI1/SNF2/SWI2 and SWI3 are suppressed by mutations in histones H3 and H4 (Kruger, Peterson et al. 1995).

Two aspects of the SWI/SNF proteins are controversial. The first is the number of SWI/SNF proteins per cell. One estimate is that there are approximately 100 copies of SWI/SNF complex in yeast cells (Cote, Quinn et al. 1994). This estimate is derived from calculations of the relative yield of a purification procedure. Another estimate is that there are at least 2000 (Wilson, Chao et al. 1996). This estimate is derived from quantitative Western data. Given the assumptions required for estimates based on recovery from purification procedures, it is likely that the estimate of 2000

molecules of SWI/SNF per yeast cell is more accurate. The second controversial aspect of the SWI/SNF complex is its association with the RNA polymerase II holoenzyme. The SWI/SNF proteins coimmunoprecipitate and copurify with the RNA polymerase II holoenzyme in one report (Wilson, Chao et al. 1996) and do not copurify with the holoenzyme in another report (Cairns, Lorch et al. 1996). It is not yet clear whether these differences result from different purification procedures or from some other reason.

Other regulators

GAL11 is a component of the RNA polymerase II holoenzyme and mediator subcomplex (Kim, Bjorklund et al. 1994). GAL11 is required for proper expression of enzymes metabolizing galactose (Suzuki, Nogi et al. 1988) and is required for appropriate transcription from a promoter containing a Ty element (Fassler and Winston 1989). Mutations in GAL11 affect a broad spectrum of genes in vivo (Fassler and Winston 1989). A mutation in GAL11 called GAL11P suppresses mutations weakening the transcriptional activator GAL4 (Himmelfarb, Pearlberg et al. 1990) apparently by creating a fortuitous interaction between GAL11 and the dimerization region of GAL4 (Barberis, Pearlberg et al. 1995).

SIN4, RGR1 (Li, Bjorklund et al. 1995) and ROX3 (Gustafsson, Myers et al. 1997) are also components of the RNA polymerase II holoenzyme and a subcomplex called the mediator. SIN4 is involved in transcription from multiple promoters, including HO, Ty, and GAL1 (Jiang and Stillman 1992). RGR1 also appears to regulate several promoters (Jiang, Dohrmann et al. 1995). Furthermore, there is a genetic interaction between RGR1 and SIN4 as overexpression of RGR1 suppresses a SIN4 deletion (Jiang, Dohrmann et al. 1995).

The presence of these genes in the RNA polymerase II holoenzyme is consistent with their involvement in transcriptional regulation in vivo but their functional role is not yet clear.

2. Other transcription factors

TATA binding protein (TBP)

TBP is a general transcription factor that binds to a promoter element called the TATA box (Sawadogo and Roeder 1985; Nakajima, Horikoshi et al. 1988). The yeast protein of 27 kD shares significant sequence homology with the human protein of 37 kD (Hahn, Buratowski et al. 1989; Hoffman, Sinn et al. 1990; Kao, Lieberman et al. 1990; Peterson, Tanese et al. 1990). The crystal structures of TBP (Nikolov, Hu et al. 1992; Chasman, Flaherty et al. 1993), TBP complexed with DNA (Kim, Nikolov et al. 1993; Kim, Geiger et al. 1993), TBP and TFIIB and DNA (Nikolov, Chen et al. 1995), and TBP and TFIIA and DNA (Geiger, Hahn et al. 1996) are available.

TBP is a good example of the evolutionary conservation among class II transcription in eukaryotes. Yeast and human TBP can substitute for each other in in vitro transcription reactions (Buratowski, Hahn et al. 1988; Cavallini, Huet et al. 1988; Flanagan, Kelleher et al. 1990; Kelleher, Flanagan et al. 1992). Consistent with the in vitro results, human TBP modified to recognize a mutant TATA box supports basal and GCN4 activated transcription in yeast cells (Strubin and Struhl 1992).

TBP is also a good example of the evolutionary conservation among class I, II and III transcription. TBP is involved in transcription by all three eukaryotic RNA polymerases; it is a component of the RNA polymerase I factor, SL1 (Comai, Tanese et al. 1992), and the RNA polymerase III

component, TFIIIB (Kassavetis, Joazeiro et al. 1992). Consistent with these observations, temperature sensitive mutations in TBP are defective in transcription by all three RNA polymerases (Cormack and Struhl 1992).

TBP binds to TBP with a kD of 2 nM with a $t_{1/2\ off}$ of approximately 50 minutes (Hahn, Buratowski et al. 1989; Hahn, Buratowski et al. 1989). TBP binds to the TATA as a monomer (Horikoshi, Yamamoto et al. 1990), bends DNA (Horikoshi, Bertuccioli et al. 1992) and binds in the minor-groove (Lee, Horikoshi et al. 1991) as confirmed by crystal structures of TBP complexed with DNA (Kim, Nikolov et al. 1993; Kim, Geiger et al. 1993). The isolation of TBP as SPT15, a suppressor of Ty element insertions, provides genetic evidence for TBP's role in binding the TATA box (Hahn, Buratowski et al. 1989).

Contacts between TBP and TFIIA and TFIIB are disrupted by alanine substitutions (Tang, Sun et al. 1996) and more radical substitutions (Bryant, Martel et al. 1996). Many of these contacts are apparent in the co-crystal structures of TBP-TFIIB (Nikolov, Chen et al. 1995) and TBP-TFIIA (Geiger, Hahn et al. 1996). TBP also binds to an affinity column of SRB2, a suppressor of CTD truncations and a component of the RNA polymerase II holoenzyme (Koleske, Buratowski et al. 1992) and co-immunoprecipitates with SPT3, a suppressor of Ty transcription and an allele-specific suppressor of a TBP mutant (Eisenmann, Arndt et al. 1992).

TBP also interacts with several activators in vitro. TBP binds directly to GAL4-VP16 affinity columns (Stringer, Ingles et al. 1990). Furthermore, the strength of TBP binding to VP16 correlates with the transcriptional activity of VP16 mutants (Ingles, Shales et al. 1991). TBP also interacts with upstream activators p53 (Truant, Xiao et al. 1993), Zta (Lieberman and Berk 1991) and

E1A (Horikoshi, Maguire et al. 1991) among others. A mutation in TBP diminishes transcriptional activation from many class II genes in yeast and can be suppressed by tethering TFIIA to TBP (Stargell and Struhl 1995). Several other mutations in TBP affect transcriptional activation (Tansey, Ruppert et al. 1994; Arndt, Ricupero-Hovasse et al. 1995; Lee and Struhl 1995).

Do the many interactions between TBP and other proteins in vitro have physiological significance? One explanation for the many interactions observed is that TBP is a sticky protein prone to artifactual interactions in vitro. On the other hand, multiple interactions with TBP are consistent with TBP's central role in the transcriptional apparatus. It is interesting to note that the interactions between TBP and TFIIA and TFIIB occur through small patches of residues. If TBP were to interact with other factors through patches of roughly the same size, it could be possible for all of the various TBP-factor interactions to occur simultaneously.

TBP also interacts with other transcriptional regulators, such as TBP associated factors (TAFs), NOTs, and NC2 as will be discussed below.

TFIIE

TFIIE has been purified as a complex of 56 kD and 34 kD proteins from humans (Inostroza, Flores et al. 1991; Ohkuma, Sumimoto et al. 1990) and as a complex of 66 and 43 kD proteins from yeast (Sayre, Tschochner et al. 1992). The sequences of the yeast subunits are similar to those of the human subunits (Feaver, Henry et al. 1994; Ohkuma, Sumimoto et al. 1991; Peterson, Inostroza et al. 1991; Sumimoto, Ohkuma et al. 1991). TFIIE associates with RNA polymerase II (Flores, Maldonado et al. 1989) as is apparent in a 2-

dimensional crystal structure (Leuther, Bushnell et al. 1996). TFIIE also associates with TFIIH (Bushnell, Bamdad et al. 1996) and stimulates its CTD kinase activity (Lu, Zawel et al. 1992; Ohkuma, Hashimoto et al. 1995). In addition to RNA polymerase II and TFIIH, TFIIE interacts with the repressor Kruppel in vitro and this interaction appears to be important for transcriptional repression (Sauer, Fondell et al. 1995).

In an in vitro system, transcription from the Adeno Major Late promoter requires TFIIE while transcription from the IgH promoter does not (Parvin, Timmers et al. 1992). These in vitro observations raise the possibility that promoters vary in their requirement for TFIIE in vivo. Generating mutations in yeast TFIIE and examining their effect on the transcription of a panel of genes would be useful in exploring this possibility.

TFIIA

Yeast TFIIA is a complex of 32 and 14 kD subunits (Ranish and Hahn 1991; Ranish and Hahn 1991). Human TFIIA is a complex of 35, 19 and 12 kD proteins (Cortes, Flores et al. 1992; De Jong and Roeder 1993; Ma, Watanabe et al. 1993; De Jong, Bernstein et al. 1995). The two larger subunits derive from a single gene product in human (De Jong and Roeder 1993; Ma, Watanabe et al. 1993) and Drosophila (Yokomori, Admon et al. 1993) TFIIA. In vitro, depletion of TFIIA from yeast (Ranish, Lane et al. 1992) and mammalian (De Jong and Roeder 1993; Ozer, Moore et al. 1994) nuclear extracts greatly diminishes basal transcription. In vivo, both subunits of yeast TFIIA are required for cell viability (Ranish, Lane et al. 1992).

TFIIA binds directly to TBP (Ranish and Hahn 1991; Usuda, Kubota et al. 1991; Cortes, Flores et al. 1992) and to the TBP-TATA complex (Buratowski, Hahn et al. 1989) where it stabilizes the interaction of TBP with the TATA box (Lee, De Jong et al. 1992). The contacts between TFIIA and TBP are evident in the co-crystal structure of TFIIA, TBP and DNA (Geiger, Hahn et al. 1996).

The addition of TFIIA overcomes repression by transcriptional inhibitors in vitro. TFIIA overcomes transcriptional inhibition by topoisomerase I (Merino, Madden et al. 1993), NC2 (Kim, Parvin et al. 1996) and HMG-1 (Ge and Roeder 1994).

In the mammalian system, TFIIA binds to the activator Zta and allows Zta to promote the binding of TFIID to the TATA box (Lieberman and Berk 1994; Ozer, Moore et al. 1994). In the Drosophila system, TFIIA enhances activation by VP16, Sp1 and NTF-1 (Yokomori, Zeidler et al. 1994). Mutations in human TFIIA diminish activation in vitro (Ozer, Bolden et al. 1996). In vivo, fusion of TFIIA to TBP rescues the activation defect of a TBP mutant (Stargell and Struhl 1995).

TFIIA has not yet been well studied in vivo. A critical issue is whether TFIIA is generally required at all promoters or plays a regulatory role at a subset of promoters. Examining the transcriptional phenotypes of conditional mutations in TFIIA subunits would be useful in exploring this issue.

3: TAFs and other TBP binding factors

TAFs

TBP is associated with factors called TBP associated factors (TAFs) in a form called TFIID (reviewed in Burley and Roeder 1996; Verrijzer and Tjian 1996; Pugh and Tjian 1992; Tanese and Tjian 1993; Goodrich and Tjian 1994). TBP and associated TAFs have been isolated from Drosophila (Dynlacht, Hoey et al. 1991), human (Pugh and Tjian 1991; Tanese, Pugh et al. 1991; Takada, Nakatani et al. 1992; Zhou, Lieberman et al. 1992) and yeast (Poon and Weil 1993; Reese, Apone et al. 1994). The composition of TFIID may not be uniform in all cell types. Human TAF105 appears to be a component of TFIID specifically in B-cells (Dikstein, Zhou et al. 1996). Like SWI/SNF and RNA polymerase II, alterations in the subunit composition of TFIID may represent a means of simultaneously regulating sets of genes.

Reconstitution of TFIID from its recombinant subunits reveals the subunit-subunit interactions between TBP and various TAFs (Weinzierl, Dynlacht et al. 1993; Chen, Attardi et al. 1994). One interesting structural feature of TAFs is the presence of histone fold motifs, which allow TAFs to form a histone octamer-like structure with other TAFs and even histones (Hoffmann, Chiang et al. 1996). A crystal structure of a heterodimer of two TAFs illustrates the contacts made between the histone folds of two TAFs (Xie, Kokubo et al. 1996). It is not yet clear whether the histone-folds in TAFs mediate protein-DNA in addition to protein-protein interactions, but it is interesting to speculate that the histone-fold TAFs bind to and alter the conformation of DNA near the TATA box.

Some Drosophila (Hoey, Dynlacht et al. 1990; Pugh and Tjian 1990; Dynlacht, Hoey et al. 1991) and human (Hoffman, Sinn et al. 1990; Smale, Schmidt et al. 1990) in vitro transcription systems reconstituted with TFIID are responsive to multiple activators. In contrast, reactions reconstituted with

TBP are not responsive to activators in these systems. Consistent with a role in activation, TAFs interact directly with activators in vitro. Human TAF155 binds to Sp1, Tat, CTF, and E1A in vitro (Chiang and Roeder 1995) and TAF32 binds to VP16 (Klemm, Goodrich et al. 1995). In vitro, Drosophila TAF150 binds to NTF-1 (Chen, Attardi et al. 1994); TAF110 binds directly to Sp1 (Hoey, Weinzierl et al. 1993); TAF60 and TAF40 bind to p53 (Thut, Chen et al. 1995); and TAF110 and TAF60 bind to bicoid (Sauer, Hansen et al. 1995). In in vitro systems, the pattern of transcriptional activation mediated by subsets of TAFs is consistent with the results of the interaction studies.

In human cells, a temperature sensitive mutation in TAF250 arrests growth at the G1 stage of the cell cycle but does not show a global defect in activated transcription (Wang and Tjian 1994). Similarly, inactivation or depletion of TAFs in yeast fails to cause global defects in class II transcription (Apone, Virbasius et al. 1996; Moqtaderi, Bai et al. 1996; Walker, Reese et al. 1996). At the non-permissive temperature, conditional mutations in different TAFs do, however, cause cells to arrest at G1, G2 and G2/M (Apone, Virbasius et al. 1996; Walker, Reese et al. 1996). Mutating TAFs in Drosophila causes defects in promoters activated by bicoid, but not other activated promoters (Sauer, Wassarman et al. 1996). These results suggest that, while TAFs are not generally required for activated transcription, they may be required for the response to certain activators. The cell-cycle phenotype of TAF mutants may indicate a requirement for TAFs for proper transcription of genes involved in regulating the cell-cycle.

TFIID and TAFs also appear to be required for initiator driven transcription. Mammalian in vitro systems reconstituted with TFIID are able to transcribe promoters with an initiator but no discernible TATA box

(Smale, Schmidt et al. 1990; Pugh and Tjian 1991; Martinez, Chiang et al. 1994). In contrast, TBP alone is unable to support transcription from these promoters. Likewise, Drosophila in vitro systems reconstituted with TAFs but not TBP alone are able to distinguish between core promoter elements in various promoters (Hansen and Tjian 1995; Verrijzer, Chen et al. 1995). Consistent with this functional requirement, Drosophila TAF150 binds directly to core promoter DNA in vitro (Verrijzer, Yokomori et al. 1994). Furthermore, studies in yeast indicate that depletion of TAFs causes transcriptional defects at the HIS3 +1 and TRP3 promoters, which contain non-consensus TATA boxes (Moqtaderi, Bai et al. 1996).

Other biochemical observations suggest additional functions for TAFs. There is some evidence that TFIID may be directly involved in recruiting and modifying general transcription factors. Human TAF250 interacts directly with RAP74, the largest subunit of TFIIF (Ruppert and Tjian 1995) and contains two kinases which phosphorylate RAP74 in vitro (Dikstein, Ruppert et al. 1996). TFIID may also be involved in modifying chromatin as TAF250 also contains a histone acetyl transferase activity (Mizzen, Yang et al. 1996). The physiological significance of these activities is not yet clear.

Other TBP associated complexes

Another complex containing TBP is called B-TFIID. This complex contains a 170 kD protein in addition to TBP, supports transcription in vitro, and contains an ATPase activity (Timmers, Meyers et al. 1992). A speculation is that the yeast equivalent to B-TFIID is a MOT1-TBP complex. MOT1 forms a complex with yeast TBP independent of TFIID (Poon, Campbell et al. 1994). It

is 170 kD, about the same size as the larger subunit of B-TFIID. MOT1 also contains an ATPase, inhibits the binding of TBP to DNA in an ATP dependent fashion, and inhibits transcription in vitro (Auble and Hahn 1993; Auble, Hansen et al. 1994). Twenty sequenced ESTs from mammals match MOT1 with a p-value less than 10^{-9} (Chao, unpublished). It will be interesting to determine whether the largest subunit of B-TFIID is a MOT1 homologue and matches some of these ESTs.

Genetic results suggest that MOT1 is not simply a transcriptional inhibitor. A mutation in MOT1 increases expression of many genes (Davis, Kunisawa et al. 1992) while other MOT1 mutations decrease the expression of many other genes (Madison and Winston 1997). In addition, MOT1 mutants show an SPT phenotype in suppressing the transcriptional effects of a delta insertion (Madison and Winston 1997). An interaction between MOT1 and TBP is supported by the observation that overexpression of TBP suppresses growth inhibition by a MOT1 dominant negative mutant (Auble, Hansen et al. 1994). What might MOT1 be doing in vivo? One possibility is that MOT1 is a specificity or recycling factor. MOT1 might help recycle TBP by removing it from TATA boxes after several rounds of transcription. MOT1 might also increase the specificity of TBP binding by removing TBP non-productively bound to non-promoter DNA.

Topoisomerase I is another factor associated with TBP. It binds TBP in a gel-shift assay and far Westerns, and substoichiometric levels co-immunoprecipitate with TBP (Merino, Madden et al. 1993). Topoisomerase I represses basal transcription and modestly stimulates activated transcription (Kretzschmar, Meisterernst et al. 1993; Merino, Madden et al. 1993). Topoisomerase activity is not required for the repressing activity, which can

be overcome by TFIIA and activators (Merino, Madden et al. 1993). It is possible that the in vitro observations of inhibition and coactivation by topoisomerase I are an artifactual result of its non-specific DNA-binding activity. There is not yet evidence that topoisomerase I influence transcription in vivo.

4. Other coactivators

Mammalian PC4 confers responsiveness to the activator GAL4-VP16 in a system reconstituted with TFIID and general transcription factors (Ge and Roeder 1994; Kretzschmar, Kaiser et al. 1994). PC4 binds directly to VP16 and a TBP-TFIIA complex in vitro (Ge and Roeder 1994). PC4 may be negatively regulated by phosphorylation in vivo as phosphorylated PC4 is transcriptionally inactive (Ge, Zhao et al. 1994). Yeast TSP1/SUB1, a protein with sequence similarity to PC4, stimulates basal transcription in vitro (Henry, Bushnell et al. 1996). TSP1/SUB1 lacks detectable coactivator activity, but binds directly to an activator in vitro (Henry, Bushnell et al. 1996). In addition to an activator, TSP1/SUB1 also binds to TFIIB (Knaus, Pollock et al. 1996). Consistent with this interaction, overexpression of TSP1/SUB1 suppresses mutations in TFIIB (Knaus, Pollock et al. 1996). However, the effect of inactivating TSP1/SUB1 on transcription in vivo has not yet been examined. It will be interesting to determine the spectrum of genes requiring TSP1/SUB1 and to examine whether TSP1/SUB1 plays a role in basal or activated transcription in vivo.

Mammalian HMG-1 and HMG-2 are members of a family of DNA-binding proteins that have been called architectural factors because of their

ability to alter the conformation of DNA (Wolffe 1994). The yeast proteins NHP6A and B are highly homologous to HMG-1 and HMG-2 (Paull, Carey et al. 1996). In an in vitro transcription reconstituted with general factors and TFIID, the addition of HMG-2 protein is required for transcriptional activation (Shykind, Kim et al. 1995). HMG-2 appears to stabilize an intermediate complex containing TFIIA, TFIIB, and TFIID bound to the TATA-box. HMG-1 also supports activation in this system (Shykind, Kim et al. 1995). In another system, HMG-1 can form a complex with TBP bound to the TATA box (Ge and Roeder 1994). However, in this system, HMG-1 represses basal transcription in vitro, an effect overcome by the addition of TFIIA (Ge and Roeder 1994). TFIIH may also be involved in overcoming repression by HMG-1 proteins. In an in vitro transcription system where TFIIH is not required, the addition of TFIIH overcomes inhibition by HMG-2 (Stelzer, Goppelt et al. 1994). Experiments with NHP6A and B in yeast cells are consistent with a role for HMG proteins in activation. Deletion of both NHP6A and B affects activated but not basal transcription from a variety of genes (Paull, Carey et al. 1996). It will be interesting to determine the promoter elements conferring a requirement for HMG like proteins and to examine whether the requirement correlates with an unusual DNA structure.

The expression of GAL4-VP16 is toxic in yeast cells (Berger, Cress et al. 1990). Mutations in the ADA genes allow yeast to survive high levels of GAL4-VP16 expression (Berger, Pina et al. 1992). Several of the ADAs form a complex that binds to activators in vitro (Silverman, Agapite et al. 1994; Horiuchi, Silverman et al. 1995). At least two ADAs, ADA2 and GCN5, appear to have counterparts in humans (Candau, Moore et al. 1996). Mutations in GCN5 diminish transcriptional activation by the activator GCN4 and the

HAP activators (Georgakopoulos, Gounalaki et al. 1995). Both yeast and human GCN5 also exhibit histone acetyl transferase activity (Brownell, Zhou et al. 1996; Wang, Mizzen et al. 1997). GCN5 acetylates histone H3 and H4 at lysines distinct from those acetylated by enzymes involved in chromatin assembly (Kuo, Brownell et al. 1996). The identification of ADA5 as SPT20 also suggests a role for the ADAs in basal transcription from a Ty promoter (Marcus, Horiuchi et al. 1996; Roberts and Winston 1996). Biochemical studies of ADAs will be helpful in determining their role in basal and activated transcription and chromatin modification.

Mammalian histone H1 is another non-specific DNA binding protein affecting transcription in vitro. A yeast homologue of histone H1 has not yet been identified, but a search of the yeast database reveals a match between histone H1 and an uncharacterized protein called LPI17 with a low but significant p-value of 10^{-6} (Chao, unpublished). In a crude system, the addition of histone H1 represses basal and activated transcription (Croston, Kerrigan et al. 1991). The addition of activators such as Sp1 and GAL4VP16 overcomes repression by H1 (Croston, Kerrigan et al. 1991). The packaging of templates in nucleosomes appears to potentiate the effects of histone H1 in this system (Laybourn and Kadonaga 1991).

Histone H1, together with the HMG proteins, topoisomerase I, and PC4, represent examples of non-specific DNA binding proteins identified based on their effect on in vitro transcription reactions. It will be interesting to determine the nature and extent of their involvement in transcriptional regulation in vivo. Given the unusual bent structure of the TBP-TATA complex, it would not be surprising if many regulatory interactions turn out to be mediated by interactions between proteins and DNA rather than

interactions between proteins. On the other hand, given the unusual conditions in in vitro transcription reactions, it would not be surprising if many of the transcriptional effects observed in vitro stem from artificially low concentrations of DNA and protein.

5. Negative regulators

Mammalian NC2, also called Dr1/DRAP1, is a factor which represses basal transcription in vitro (Meisterernst and Roeder 1991; Inostroza, Mermelstein et al. 1992; Mermelstein, Yeung et al. 1996). Human NC2 is a complex of two subunits with molecular weights of 19 and 22 kD, both with histone fold motifs. In yeast, NC2 contains subunits with similar sequences (Goppelt and Meisterernst 1996; Gadbois, Chao et al. 1997; Kim, Na et al. 1997). NC2 binds directly to TBP (Meisterernst and Roeder 1991; Inostroza, Mermelstein et al. 1992; Gadbois, Chao et al. 1997). More specifically, NC2 competes with TFIIA for binding to TBP (Kim, Zhao et al. 1995) and prevents the association of TFIIB with promoter-bound TBP (Goppelt, Stelzer et al. 1996). In vitro, TFIIA overcomes the repression by NC2 in a promoter specific fashion (Kim, Parvin et al. 1996).

Genetic evidence suggests that NC2 is a global negative regulator of class II transcription. NC2 alleviates the global defects in mRNA synthesis caused by a mutation in the RNA polymerase II holoenzyme component SRB4 (Gadbois, Chao et al. 1997). The presence of histone folds in the TBP-associated proteins NC2 and TAFs is intriguing. These results are consistent with models in which the activity of TBP is modulated by interactions of its

associated proteins with DNA. The structure of a TBP-NC2 complex is likely to reveal an unusual architecture with significant regulatory implications.

The NOTs are a collection of four genes whose mutation increases the transcription of a subset of genes (Collart and Struhl 1993; Collart and Struhl 1994). There are several genetic interactions between the NOTs, TBP, MOT1 and SPT3. Mutations in TBP or SPT3 suppress a NOT mutation (Collart 1996). In addition, NOT mutations suppress the toxicity of TBP overexpression in a MOT1 mutant background (Collart 1996). An understanding of the role of the NOTs in transcriptional regulation awaits biochemical experiments that take advantage of the genetic clues obtained so far.

6. Promoter elements

A typical eukaryotic promoter contains a TATA box, an initiator, and regulatory elements. The TATA box is a loosely conserved sequence element rich in thymidine and adenine (Breathnach and Chambon 1981). In eukaryotes, the TATA box is typically 40 bp upstream of the transcription start site in yeast and 25 bp upstream in mammals (Li, Flanagan et al. 1994). The initiator (Inr) is a sequence element approximately 17 bp in length, located at the transcription start site (Smale, Schmidt et al. 1990). Outside of model promoters such as the Adeno Major Late and the terminal deoxynucleotyidyl transferase gene, both the TATA box and Inr elements are imprecisely defined. Yeast Upstream Activating Sequences (UASs) and eukaryotic enhancers serve as binding sites for gene-specific transcriptional activators and repressors. Enhancers can function at kilobase distances upstream or downstream of the transcription start site (Banerji, Rusconi et al. 1981).

The availability of sequence information from the yeast genome project and the development new technologies for examining transcription on a genome-wide scale provide an opportunity to improve our understanding of promoter sequence elements. The entire set of yeast promoters can be compared to identify and define important functional elements whose presence or absence can be correlated with relative differences in transcript levels. This information could be used to develop what Eric Lander has called "a periodic table" of promoter sequence elements. Such a periodic table would be useful in identifying patterns and sequence elements in promoters and in predicting the behavior of promoters under different physiological conditions. There are at least two challenges that need to be overcome for this analysis. The first is ignorance of the position transcription start sites. Few start-sites have been mapped in yeast, and it is likely that useful comparisons will require accurate definitions of the downstream end of promoters. The second challenge is the lack of appropriate computer algorithms to identify weak consensus sequences. These challenges do not seem insurmountable, and there is little doubt that a "periodic table of promoters" would provide profound insights into transcriptional regulation.

7. Chromatin

Besides the in vitro work discussed above, there is a great deal of in vivo work implicating chromatin in transcriptional control. Depletion of histones in vivo increases the transcription from many promoters, consistent with a role for nucleosomes in repressing transcription (Han and Grunstein 1988). The N-termini of histones H2A, H2B, and H3 and H4 are required for repression of basal transcription (Lenfant, Mann et al. 1996). The N-terminus of histone H4 also appears to play a role in transcriptional activation since

mutations in its N-terminus decrease activation at the GAL1 and PHO5 promoters (Durrin, Mann et al. 1991). Over or under-expression of either histone H2A and H2B give rise to subtler effects. Both changes alter transcription from mutant promoters with a delta element insertion (Clark-Adams, Norris et al. 1988). Other genetic results suggest an interaction between chromatin and components of the RNA polymerase II holoenzyme. Mutations in histone H2A and B suppress mutations in SNF2/SWI2, and SNF5, and 6 (Hirschhorn, Brown et al. 1992), and mutations in histone H3 and H4 suppress mutations in SWI1/SNF2/SWI2 and SWI3 (Kruger, Peterson et al. 1995).

In addition to the SWI/SNF proteins, two other chromatin remodeling activities have been described. Drosophila NURF is an ATP-dependent chromatin remodeling activity containing four subunits (Tsukiyama and Wu 1995). One of these subunits, ISWI, is closely related to yeast SWI2/SNF2 (Tsukiyama, Daniel et al. 1995). Yeast RSC is another ATP-dependent chromatin remodeling activity (Cairns, Lorch et al. 1996). Like NURF, RSC also contains a homologue of SWI2/SNF2 (Cairns, Lorch et al. 1996). RSC also contains at least three other proteins which are related to components of the SWI/SNF complex (Cairns, Lorch et al. 1996). Whether these chromatin remodeling complexes play a role in transcriptional regulation is not yet clear.

Chapter 2: Isolation and characterization of complex containing SRBs

Background

In the fall of 1992, there were several clues to the function of the CTD and SRB proteins. Charles Scafe and I had extended Allison and Ingles' work demonstrating the involvement of the CTD in transcriptional activation (Allison and Ingles 1989; Scafe, Chao et al. 1990). Craig Thompson had evidence that the various SRBs were allele-specific suppressors of CTD truncation mutants. These genetic results suggested a close association between the SRBs and CTDs. At the same time, Tony Koleske had preliminary evidence from immunoprecipitation experiments that the SRBs were associated with one another and was in the process of purifying what later turned out to be the holoenzyme.

Because Craig Thompson had identified factors interacting with the CTD genetically, I set out to complement his work by identifying factors interacting with the CTD biochemically.

Results and Discussion

To identify proteins binding to the CTD, I applied a yeast extract to a CTD affinity column (see Figure 7 in Thompson, Koleske et al. 1993). I found that SRB2, SRB4, SRB5 and SRB6 bound specifically to the CTD column. These biochemical results demonstrated an interaction between the SRBs and the CTD and reinforced the genetic results obtained by Thompson and Koleske in their suppression analysis.

I next set out to develop an assay for CTD binding proteins. I applied a nuclear extract to a CTD affinity column and found that it was depleted of

transcriptional activity. Activity was restored when I added back material bound to the column. With this assay in hand, I set out to purify the reconstituting activity, which I suspected would contain the SRB proteins.

The reconstituting activity was purified over a CTD affinity column and two ion exchange columns (See Figure 4, Wilson, Chao et al. 1996). As expected, the activity coeluted with a complex containing SRB proteins and CTD kinase activity. These results extended Thompson and Koleske's results with SRB2 and SRB5 in vitro by demonstrating a requirement for a larger complex of SRB proteins for transcription in vitro. This requirement for SRB proteins in vitro was a prelude to Craig Thompson's later work demonstrating a requirement for SRB proteins for transcription in vivo (Thompson and Young 1995). Similarly, the observation of CTD kinase activity in the SRB complex was a prelude to Liao and Zhang's identification of SRB10/11 as a kinase-cyclin pair.

I next set out to determine the function of the SRB complex in activated transcription. The depletion-reconstitution assay was not useful for this purpose because there was no basal activity in the absence of SRBs. I spent the next year developing a reconstituted in vitro system. I obtained RNA polymerase II from Sha-Mei Liao and recombinant TBP and native TFIIE from Tony Koleske. Michelle Sonu and I prepared recombinant TFIIB, and I purified TFIIF and TFIIH.

When added to this reconstituted system, the SRB complex had a modest affect on basal transcription. However, despite much effort, I was unable to observe activated transcription in this system. In the meantime, Tony Koleske had finished purifying the SRB complex and found that he had isolated an RNA polymerase II holoenzyme responsive to activators. A little

later, Kim and Kornberg isolated the mediator, an SRB containing complex which supported activated transcription in vitro (Kim, Bjorklund et al. 1994). The mediator was dissociated from RNA polymerase II with a monoclonal antibody directed against the CTD and subsequently purified by ion-exchange chromatography. While both the mediator and SRB complex purified by CTD affinity chromatography share at least some subunits, the extent of their similarity is not yet clear.

In summary, my work on the SRB complex demonstrated a physical association between the SRBs and the CTD, identified a multisubunit complex containing SRBs, and demonstrated a requirement for the SRBs in transcription in vitro.

Chapter 3: Identification of SWI/SNF proteins as components of the SRB complex

Background

In the fall of 1995, the Green, Kingston and Peterson labs had isolated complexes containing SWI/SNF proteins and shown that they could remodel chromatin in vitro (Cote, Quinn et al. 1994; Imbalzano, Kwon et al. 1994; Kwon, Imbalzano et al. 1994). These biochemical results were supported by a great deal of genetic work implicating the SWI/SNF proteins in transcriptional activation and chromatin remodeling (work from Herskowitz, Winston, and Carlson labs reviewed in Winston and Carlson 1992; Peterson and Tamkun 1995).

Tony Koleske and the Kornberg lab had both purified RNA polymerase II holoenzymes and shown that they were responsive to activators in vitro. Because the SRBs appeared to play a key role as a hallmark of the holoenzyme, I set out to identify other proteins in the SRB complex.

Results and Discussion

I scaled up the purification procedure for the SRB complex and took three approaches to identifying other proteins in the complex. First, I tried to obtain microsequence data from the individual proteins in collaboration with Bill Lane. These efforts were unsuccessful probably because the samples were poorly resolved by SDS-PAGE; in a sample with such a large number of bands, loading sufficient amounts of proteins caused smearing.

Next, I prepared polyclonal antibodies against the entire complex. Chris Wilson used these antibodies to screen a phage expression library and identified several SWI2/SNF2 as a reactive antigen. He later confirmed the presence of SWI2/SNF2 and other SWI/SNF proteins in the SRB complex and in the holoenzyme (Wilson, Chao et al. 1996). Tony Imbalzano and Bob Kingston confirmed that both the SRB complex and the holoenzyme contained the expected chromatin remodeling activity. The identification of the SWI/SNF genes as components of the holoenzyme provides an explanation for how chromatin remodeling activity is targeted to promoters.

Finally, I identified some of the proteins by mass-spectrometry. I did some preliminary work with Paul Matsudaira and successfully identified an abundant CTD-binding protein and several subunits of mammalian TFIIH. Later, I collaborated with Jun Qin and Brian Chait to identify proteins in the SRB complex with a new mass spectrometric technique. Qin and Chait had developed a new method for identifying proteins with an instrument called a Matrix Assisted Laser Desorption/Ionization Ion Trap or MALDI Ion Trap (Qin and Chait 1996; Qin, Ruud et al. 1996). In collaboration with the Chait lab, Chris Wilson and I identified several additional proteins in the SRB complex (Qin et al., submitted). The identified proteins included SRB4, SRB5, RGR1, and SIN4, proteins previously shown to be components of the RNA polymerase II holoenzyme. The identified proteins also included SUB1, a yeast homologue of the mammalian coactivator PC4, and UME6, a Zn-finger protein, as well as 3 uncharacterized yeast proteins. SUB1, UME6 and the 3 other proteins have not yet been confirmed as components of the SRB complex by other techniques. The mass-spectrometric technique was rapid and sensitive but gave many false negatives. Despite indications of sufficient

quantity by multiple staining methods, many proteins did not give signals in the mass-spectrometer for unknown reasons.

In summary, my work identifying the SWI/SNF protein and other proteins in the SRB complex helped link the SRBs and holoenzyme function to chromatin remodeling and coactivation.

Chapter 4: Isolation of a mammalian SRB gene and mammalian RNA polymerase II holoenzyme

Background

In the fall of 1993, many believed that transcription in mammals was fundamentally different from transcription in yeast. There were two apparent differences. The first was a failure to observe TAFs in yeast. Buratowski and Sharp purified TBP from yeast, and it appeared to be monomeric rather than associated with TAFs (Buratowski, Hahn et al. 1988). Biochemical evidence suggested that a role for TAFs and TFIID in activated transcription in Drosophila and humans. Yeast seemed to be fundamentally different in lacking TAFs. The second difference was a failure to observe a mammalian version of the RNA polymerase II holoenzyme. The yeast RNA polymerase II holoenzyme appeared to play a role in activated transcription. In contrast, higher eukaryotes seemed to be fundamentally different in lacking SRBs. To address this issue, our lab sought to identify and isolate a mammalian RNA polymerase II holoenzyme.

Results and Discussion

I first attempted to purify a mammalian SRB complex by using the CTD affinity chromatography procedure that had been effective in purifying yeast SRBs. I was able to purify a complex that bound specifically to the CTD, but I was unable to purify enough to obtain microsequence information. Probably because of the small amounts available, Jeff Parvin was also unable to obtain coactivating activity from the CTD-binding complex.

I next tried to purify large complexes containing mammalian RNA polymerase II. I was successful in detecting large RNA polymerase II complexes by gel filtration but had great difficulty in purifying them.

In parallel, I was trying to clone mammalian homologues of SRB genes by database searching. Doug Bassett and Phil Hieter had just set up an automated program for screening expressed sequence tag (EST) databases on a monthly basis. A few months after we set it up, the program notified me of a match between a newly sequenced EST and yeast SRB7. Peter Murray and I cloned the full length version of this gene, which we called hSRB7 (See figures in Chao, Gadbois et al. 1996). Ellen Gadbois and I checked whether hSRB7 could complement a deletion of the yeast SRB7 gene. It could not, but we found that chimeras of human and yeast SRB7 could complement the deletion. Michelle Sonu and I demonstrated that mammalian SRB7 binds to a CTD affinity column, as expected from work with yeast SRB7. Finally, using antibodies raised against mammalian SRB7, I isolated a complex containing mammalian SRB7, RNA polymerase II and substoichiometric TFIIE and TFIIH. Steve Anderson and Jeff Parvin helped characterize the mammalian holoenzyme complex with functional assays.

A few months before we published our work (Chao, Gadbois et al. 1996), Ossipow and Schibler used a monoclonal antibody directed against TFIIH to isolate a mammalian holoenzyme complex containing RNA polymerase II and all the general transcription factors (Ossipow, Tassan et al. 1995). Later, Vincent Ossipow used our antibodies to show that hSRB7 was also a part of this immunoprecipitated complex. A few months after we published our work, Maldonado and Reinberg used a monoclonal antibody directed against TFIIF to isolate a mammalian holoenzyme complex containing RNA

polymerase II, TFIIE, F and H, and hSRB7 (Maldonado, Shiekhattar et al. 1996).

What accounts for the different forms of mammalian holoenzyme? One reason could be destabilization of the holoenzyme by chloride ions. Michelle Sonu and I found that chloride can dissociate yeast SRBs from a CTD column. Other anions, such as sulfate or acetate, did not have this effect. Similarly, mammalian proteins were dissociated from a CTD column by chloride but not other anions. Because chloride salts are typically used when purifying mammalian transcription factors, their destabilizing effect may have hampered the isolation of intact holoenzymes. The destabilizing effect of chloride may have also facilitated the purification of yeast TBP and delayed the isolation of yeast TAFs. Yeast TAFs are dissociated from a TBP column by chloride but not acetate (Reese, Apone et al. 1994). Buratowski and Sharp were probably able to purify monomeric yeast TBP relatively easily because the chloride used in their purification procedure dissociated TBP from TAFs and other proteins (Buratowski, Hahn et al. 1988).

After the isolation of yeast TAFs by Reese and Green and Poon and Weil (Poon and Weil 1993; Reese, Apone et al. 1994) and the isolation of a mammalian RNA polymerase II holoenzyme by us and others, most in the field became convinced that transcription is fundamentally the same in yeast and mammals. However, some differences are still claimed. First, no examples of the proline-rich or glutamine-rich type activators found in higher eukaryotes have been reported in yeast. The classes of activators are defined based on sequence composition rather than a functional test. Because the definition of activator classes is vague, it is not yet clear whether the lack of proline-rich and glutamine-rich activators in yeast is a significant

difference. Second, the existence of a histone H1 homologue in yeast has not yet been demonstrated. Histone H1 appears to play an important role in chromatin packaging and transcriptional regulation in higher eukaryotes so it would be surprising if yeast lacked H1. However, there is an uncharacterized gene with homology to histone H1 in the yeast database. LPI17 matches histone H1 with a p-value of 10^{-6} (Chao, unpublished). It will be interesting to determine whether LPI17 represents a yeast homologue of histone H1.

Chapter 5: Purification of yeast NC2

Background

In the summer of 1995, repressors had been well studied biochemically but not genetically. Inostroza and Reinberg had described a TBP-binding repressor called Dr1 (Inostroza, Mermelstein et al. 1992). Ge and Roeder had described another protein with repressing activity called HMG-1 (Ge and Roeder 1994). Besides histones, nothing was known about general repression in vivo or in yeast.

In our lab, Craig Thompson had shown that the SRBs were generally required for transcription in vivo. A temperature sensitive mutation in SRB4 was shown to shut-down transcription rapidly at the non-permissive temperature (Thompson and Young 1995). Ellen Gadbois sought to identify factors interacting with SRB4 by isolating suppressors of this ts mutation.

Results and Discussion

One of the SRB4 suppressors, which was later named NCB1, contained a histone-fold motif. Because histones had been isolated in the SPT screen, Ellen Gadbois contacted Fred Winston to determine whether NCB1 represented an unpublished SPT gene. Ellen Gadbois then learned that NCB1 had not been isolated in the SPT screen but had been isolated by Greg Prelich in a screen for mutations bypassing the requirement for an upstream activating sequence. Because TAFs and other coactivators had been shown to bind to TBP, Ellen Gadbois collaborated with Joe Reese and Michael Green to determine whether NCB1 bound to a TBP affinity column. NCB1 did in fact

bind to TBP. We soon learned that Danny Reinberg's lab had also isolated yeast and mammalian NCB1 as proteins associated with the TBP-binding repressor Dr1. It was also reported that Dr1/DRAP1 was equivalent to Meisterernst and Roeder's repressor NC2.

We now suspected that the SRB4 suppressor was the yeast homolog of a mammalian negative regulator. While Ellen Gadbois performed genetic experiments to confirm this notion, I began to study NCB1 in vitro. Using TBP affinity column eluates from Joe Reese, I purified a complex containing NCB1 and another protein (Gadbois, Chao et al. 1997). Ellen Gadbois confirmed that this complex contains DRAP1 and Dr1 by Western blotting. For simplicity, we decided to call this complex yeast NC2, with the large subunit named NCB1 and the small subunit named NCB2.

The material from the TBP-affinity column was insufficient for functional assays. I attempted to purify yeast NC2 from crude extracts but was unsuccessful. I next tried to purify an epitope-tagged version of NC2 and was successful. I then used this material to confirm that NC2 was able to repress the transcriptional activity of the yeast RNA polymerase II holoenzyme in vitro.

Our work was significant in demonstrating the conservation of NC2 from yeast to humans and in demonstrating the physiological relevance of repression by NC2. Ellen Gadbois' genetic work showed that NC2 was a general negative regulator of transcription in vivo.

Chapter 6: Discussion

Models for assembly of the preinitiation complex

In vivo, how much of the preinitiation complex is assembled on the TATA box and how much is assembled off of DNA? At one extreme, transcription factors are proposed to assemble in a stepwise fashion. The plausibility of this pathway has been demonstrated in vitro (Buratowski, Hahn et al. 1989). At the other extreme, a subset of the transcription factors is proposed to pre-assemble in a holoenzyme complex before final assembly on the TATA box. The plausibility of this pathway has also been demonstrated in vitro (Koleske and Young 1994). SRBs are generally required for transcription in vivo (Thompson and Young 1995), but this general requirement does not address the issue of whether the SRBs and RNA polymerase II assemble on the DNA or off the DNA.

Whether transcription factors assemble sequentially or are preassembled, their association and dissociation are likely to be dynamic. The number of TBP molecules in the cell is estimated to be approximately 50,000 by quantitative Westerns (Tony Lee, unpublished). This number of TBP molecules is sufficient to allow TBP to remain stably bound at all promoters in the genome. Consistent with this notion, TBP appears to remain bound to the TATA box after RNA polymerase II clears the promoter in an in vitro system (Zawel, Kumar et al. 1995). The number of holoenzyme molecules is estimated to be approximately 2,000 to 4,000 per cell and the number of RNA polymerase II molecules approximately 20,000 per cell (Koleske and Young 1994). These numbers are consistent with a cycle in which SRBs dissociate

from RNA polymerase II at some point after initiation and reassociate at some point before reinitiation.

The sequential assembly model and the holoenzyme model represent two extremes in a spectrum of possibilities. Much as different promoters require different subsets of the basal transcription factors and are influenced to varying degrees by various regulatory factors, it is possible that the transcriptional apparatus assembles by different pathways at different promoters.

Models for transcriptional activation

The various models for transcriptional activation can be classified into three categories: recruitment, isomerization, and modification. I discuss these models in the context of transcription initiation although it is clear that regulation can occur at other steps in the transcription cycle (Yankulov, Blau et al. 1994; Akhtar, Faye et al. 1996).

In the recruitment class of models, activators increase the rate of transcription by recruiting components of the transcriptional apparatus to promoters (Ptashne and Gann 1997). Any means of recruitment suffices for activation in this model. Recruitment might occur through protein-protein interactions. The many interactions between activation domains and various general transcription factors and coactivators may reflect genuine interactions involved in recruitment in vivo. Transcriptional stimulation by artificial recruitment of transcription factors, such as GAL11 (Barberis, Pearlberg et al. 1995) and TBP (Chatterjee and Struhl 1995; Klages and Strubin 1995), is also consistent with the recruitment model. Recruitment might also occur

through protein-DNA interactions. Architectural cofactors such as HMG proteins change the conformation of DNA to recruit additional proteins to the preinitiation complex via protein-DNA interactions (Shykind, Kim et al. 1995). It is interesting to note that many purified coactivators such as HMG1 and 2, topoisomerase I, and PC4, are non-specific DNA binding proteins.

In the isomerization class of models, activators increase the rate of initiation by inducing a conformational change in the preinitiation complex. This might be a change in a protein, such as TFIIB, (Roberts and Green 1994) or it might be a change in the structure of promoter DNA. The closed to open transition of promoters is a target for activators, and a similar transition has been observed in mammalian extracts (Wang, Carey et al. 1992; Wang, Gralla et al. 1992).

In the modification class of models, activators increase the rate of initiation by inducing a post-translational modification of a component of the preinitiation complex. The activity of at least two kinases, SRB10 and KIN28, is essential for appropriate transcriptional regulation in vivo (Cismowski, Laff et al. 1995; Liao, Zhang et al. 1995). Kinase (Dikstein, Ruppert et al. 1996) and acetylase (Brownell, Zhou et al. 1996; Mizzen, Yang et al. 1996) activities have also been detected in several other transcription factors. The exact role of these enzymatic activities in transcriptional regulation is not yet clear but they are likely to play an important role.

The three models of transcriptional activation are not mutually exclusive. It is likely that transcriptional regulation involves multiple mechanisms whose contributions vary from promoter to promoter. From an evolutionary perspective, a diversity of regulatory mechanisms makes sense if one considers promoters to be "selfish." If an individual promoter's

objective is to express its gene at the appropriate level and the promoter competes with all other promoters in the genome to accomplish this objective, one might imagine that promoters would evolve different mechanisms to accomplish this goal. Promoters would evolve to use any means available to ensure their proper expression. Because there would be little evolutionary pressure for promoters to use the same mechanisms, a diversity of regulatory mechanisms would result. The differential sensitivity of promoters to particular mutations in vivo or to particular biochemical manipulations in vitro argues that diverse regulatory mechanisms are at work. Careful study of the details of transcriptional regulation at a few model promoters will most likely provide multiple examples of the three classes of regulatory mechanisms.

Conditions in vivo vs. in vitro

What is the nature of the milieu inside the eukaryotic nucleus? By weight, a cell is estimated to be 70% water, 20% protein and 1% DNA. If the density of water, protein and DNA is approximated at a 1 g/ml, then the concentration of protein in the cell is approximately 200 mg/ml. If the concentration of protein is assumed to be uniform throughout the cell, then the concentration of protein in the nucleus is approximately 200 mg/ml. If the nucleus is estimated to be 10% the volume of the cell, the concentration of DNA is approximately 100 mg/ml (10 mg DNA/1 ml/0.1 cell volume=100 mg DNA/ml). The concentration of DNA and protein in the nucleus approximates the concentration of proteins in crystals, which are approximately 50% water. Principles gleaned from studying the behavior of the dilute protein solutions in in vitro reactions are not likely to apply to the

behavior of proteins in the concentrated environment of the cell. A better understanding of the biophysical behavior of concentrated solutions of protein and nucleic acids would greatly aid the design of experiments designed to recreate conditions in vivo.

How do standard transcription reactions compare with the conditions in vivo? Estimates of the relative concentrations of RNA polymerase II and template DNA in vivo and in vitro are revealing. If the average yeast cell is assumed to be a sphere with a diameter of 5 μm, the volume of a yeast cell is approximately 1 picoliter. If the nucleus is 10% the volume of the cell, then the volume of the nucleus is 100 femtoliter. There are an estimated 20,000 molecules of RNA polymerase II in the cell (Koleske and Young 1994). Based on these estimates, the concentration of RNA polymerase II is approximately 400 nM. What about promoters? The yeast genome ($14X10^6$ bp of DNA) contains 5000 genes. If we assume 1 TATA box per gene, then 0.2% of the yeast genome is a binding site for the transcriptional apparatus (5000 genes X 1 TATA box/gene/5 bp/TATA box / $14 X 10^6$ bp=0.0018). The concentration of TATA boxes in the nucleus is approximately 100 nM.

These estimates of polymerase and promoter concentrations are higher than the amounts in typical in vitro transcription reactions. If the typical reaction contains 500 ng of RNA polymerase II and 100 ng of DNA in a volume of 20 μl, the concentration of RNA polymerase II in these reactions is approximately 10 nM and the concentration of TATA boxes 1 nM. By these calculations, there is a forty-fold lower concentration of RNA polymerase II and a hundred fold lower concentration of promoters in in vitro transcription reactions. Given these large differences, it is difficult to predict

what effect extreme dilution would have on the behavior of transcription factors in vitro.

Besides concentration, another problem with in vitro transcription reactions is their failure to recreate the internal architecture of the nucleus. Some transcription factors are localized and concentrated in subnuclear compartments. For instance, phosphorylated RNA polymerase II localizes to speckles (Bregman, Du et al. 1995). Likewise, DNA may be packaged into special domains or packaged into special forms of chromatin.

Large protein complexes

There are many examples where factors dedicated to a common function are organized in large complexes. The ribosome, spliceosome and signal recognition particle are groups of translation, splicing and translocation factors organized in megadalton-sized complexes. Because a feature shared by these three large complexes is the presence of structural RNAs, an interesting speculation is that the RNA polymerase II holoenzyme might also contain a structural RNA. Other processes once thought to involve the interaction of proteins in solution may also involve large complexes. Recently, a large complex of kinases involved in a signal transduction pathway has been described (Choi, Satterberg et al. 1994).

Why might proteins be organized in large complexes? The need for rapid and efficient catalysis may be one reason. In the case of RNA polymerase II, proteins, nucleotides and DNA must be brought together to produce mRNA. Organization of these macromolecules in an optimized configuration is likely to increase the speed and efficiency at which catalysis is performed.

Assembling macromolecules in an organized structure reduces inefficient side reactions from non-productive interactions and increases the speed of reactions relative to random diffusion and collision. Given the extremely concentrated environment of the cell, these considerations may be extremely important for catalytic efficiency.

Another reason for large complexes may be the need to process regulatory signals. An apparatus like the RNA polymerase II holoenzyme modulates its catalytic output in response to multiple regulatory inputs. These inputs must be processed by many sequential and concurrent protein-protein interactions and post-translational modifications to achieve the appropriate output. It is likely that the degree of processing that is possible is roughly proportional to the size of the complex involved. For highly regulated processes like transcription, megadalton-sized complexes, like the RNA polymerase II holoenzyme, are likely to represent an evolutionary response to the requirements of processing multiple regulatory signals.

In an extreme view, one might imagine that the large complexes themselves are part of a higher order architecture. For example, an extracellular signal could alter gene expression via a cascade of physically and catalytically coupled events. Signals would be transduced through a complex of physically associated kinases, which would activate the RNA polymerase II holoenzyme to increase the transcription of target genes. The holoenzyme would then transfer the induced mRNAs to processing complexes such as the spliceosome, which would in turn transfer the mRNA to tracks. These tracks would deliver the mRNA to waiting ribosomes for the production of the required proteins. In this speculative pathway, efficiency is increased by

physically coupling the large protein complexes mediating signal transduction and gene expression.

Much as the identification and characterization of individual subunits are prerequisites for an understanding of large multisubunit complexes, the identification and characterization of large multisubunit complexes are prerequisites for an understanding of the higher order architecture inside cells. The isolation and characterization of RNA polymerase II holoenzymes thus provide valuable starting points for studies of the higher order structures involved in transcriptional regulation.

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Appendix: Reprints

Arranged alphabetically by author and listed below in reverse chronological order

Qin, J., D. Fenyo, Y. Zhao, W.W. Hall, D.M. Chao, C.J. Wilson, R.A. Young, B.T. Chait (Submitted). "A strategy for rapid, high confidence protein identification."

Scully, R., S.F. Anderson, D.M. Chao, W. Wei, L. Ye, R.A. Young, D.M. Livingston, J.D. Parvin (In press). "BRCA1 is a component of the RNA polymerase II holoenzyme." Proceedings of the National Academy of Sciences.

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Activation without a vital ingredient

David M. Chao and Richard A. Young

The complicated machinery that initiates transcription of protein-coding genes contains components, known as TAFs, that are held to be generally required for gene activation. Experiments *in vivo* change that view.

REGULATION of gene expression is fundamental to living processes, and much of it occurs at the level of transcription initiation. One form of regulation, called gene activation, produces a marked increase in the rate at which messenger RNAs are copied from the gene. The textbook model of gene activation, derived from studies *in vitro*, is that gene-specific activators bind to TBP-associated factors (TAFs), a set of proteins that associate with TATA-binding protein (TBP), thereby recruiting this component of the transcriptional machinery to promoter DNA[1,2]. However, new evidence, reported by groups led by Michael Green and Kevin Struhl on pages 185 and 188 of this issue[3,4], demonstrates that gene activation can occur in living cells in the absence of functional TAFs. Does the textbook need revision?

Eukaryotic cells contain many thousands of protein-coding genes, and the task of transcribing them into messenger RNA falls to RNA polymerase II. Genes transcribed by RNA polymerase II (known as class II genes) have several promoter elements that are necessary for regulated transcription initiation. Most promoters contain a sequence element called a TATA box (because it is rich in thymine and adenine) that acts as the binding site for TBP and the rest of the transcription-initiation apparatus. The rate of transcription of each gene is regulated by activator proteins that bind to promoter DNA sequences called enhancers, generally located upstream of the TATA box. Activators, which typically have a DNA-binding domain and an activating domain, are thought to stimulate gene expression by interacting with components of the transcription-initiation apparatus. As with many other biological processes, our understanding of this is due to the combined efforts of biochemists and geneticists (see box overleaf).

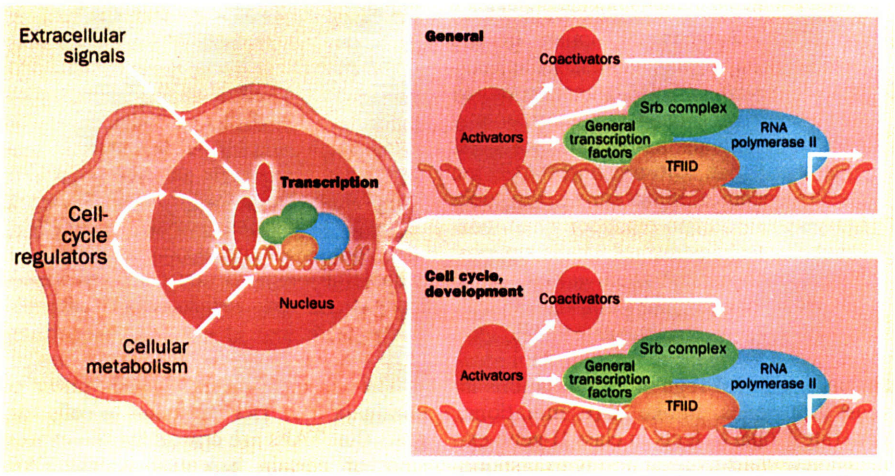
Several important features of transcriptional regulation have been re-created in the test tube with purified RNA polymerase II and a set of proteins called general transcription factors[1,2]. These include TFIID (a complex of TBP plus TAFs), TFIIA, TFIIB, TFIIF, TFIIE and TFIIH, which are evolutionarily conserved between yeast and humans. When RNA polymerase II and general transcription factors are added to a naked DNA template, transcription can initiate at the appropriate promoter site. The addition of an activator can, under certain conditions, increase the level of RNA synthesis.

The TAFs have been implicated in transcriptional activation *in vitro* because activators can stimulate transcription in some systems reconstituted with mammalian RNA polymerase II and the general transcription factors, but not when TBP is substituted for TFIID (refs 1, 2). The model derived from these studies is that activators bind to the TAF proteins directly, thereby recruiting the TFIID complex to promoter DNA. Support for this model comes from experiments in which the response to various activators can be reconstituted with specific recombinant TBP–TAF subcomplexes. In systems reconstituted with highly purified mammalian components, cofactors such as PC4 and HMG2 are required in addition to TFIID to obtain activation *in vitro*.

The studies described by Green, Struhl and their colleagues[3,4] were designed to find out whether yeast TAFs are generally required for class II gene expression and regulation *in vivo*. Three different experimental approaches were used to inactivate or substantially reduce the levels of TAFs in cells. In one, conditional mutations in specific TAFs were employed; these mutant TAFs can function appropriately at one temperature, but not another. The other approaches involved use of molecular-genetic techniques to shut down TAF synthesis and cause TAF degradation.

In each of the experiments, TAF inactivation or elimination led to cell death. Surprisingly, however, the dying cells were not compromised in gene expression generally. Moreover, activation was unaffected at all of the promoters tested. In contrast, mutations in most other components of the transcription apparatus have dramatically deleterious effects on class II gene expression *in vivo*. The new results raise two important questions. What factors are required for gene activation at class II promoters if the TAFs are not? And what do TAFs do *in vivo*?

Recent genetic and biochemical evidence indicates that transcription is initiated *in vivo* by a large complex of proteins called an RNA polymerase II holoenzyme[5,6]. This apparatus has been isolated from yeast and mammalian cells, and consists of RNA polymerase II, a subset of general transcription factors and a subcomplex containing many regulatory proteins. Among these regulatory proteins are Srbs, which were genetically identified



Regulation of gene expression. Signals from outside and inside the cell influence the rate of transcription of class II genes. This is achieved by activators or other transcriptional regulators, which are believed to act through components of the RNA polymerase II holoenzyme, coactivators and TFIID (a complex of TATA-binding protein and associated factors known as TAFs). The involvement of TAFs is now called into question by the experiments of Green, Struhl and colleagues[3,4], discussed here, which show that TAFs are not generally required for gene activation *in vivo*. Rather than playing a general role, TFIID may be required for the activation of genes that participate in controlling the cell cycle and development.

Biochemistry and genetics: checks and balances

To study such complicated phenomena as transcriptional activation, biologists turn to two tried and true approaches — the biochemical and the genetic.

Biochemists use a strategy of reconstruction. They seek to reconstitute biological processes in test tubes. The first step is to develop an assay that reveals the activity of interest. The second is to purify the components of the cell necessary to reconstitute that activity from crude cell extracts. Finally, the purified proteins are studied individually and together to determine what each one does. Most of the three-dozen proteins required for transcription of class II genes were identified biochemically[2,10,11].

Geneticists, on the other hand, employ a strategy of destruction. They disrupt biological processes by making mutations and examining the consequences in living cells. The first step is to develop a screen for cells exhibiting disruption in the process being studied (transcription, pattern formation, signal transduction or whatever). The second is to pinpoint any genetic mutations associated with that disruption, then further characterize the genes concerned to determine the functions of their protein products. The fundamental mechanisms of transcriptional regulation in bacteria were worked out in this way[12].

But each approach has its limitations. Reconstituted biochemical reactions are usually a highly simplified version of what goes on inside a living cell. The reaction may be missing essential features of the process as it occurs *in vivo* (which may be poorly defined) or it may harbour hidden flaws — for example, TFIIC is missing from the roll call of general transcription factors because its identification was an artefact of the *in vitro* assay conditions.

The problem for geneticists is that the genes identified in genetic selections may not always be directly involved in the process of interest. Thus, the general transcription factor TFIIB was inadvertently identified in yeast by a screen intended to isolate translation factors. Other transcription factors have been isolated in screens for cell-cycle regulators because of their indirect effects on the expression of these regulators.

Some of these drawbacks can be overcome by sharing tactics, as we have seen in the transcription story. The chief weakness of biochemistry — inability to discriminate between what is real and what is artefact — can be addressed genetically. A biochemist with a newly purified enzyme can mutate the gene encoding that enzyme. If the biochemist is correct about the enzyme's activity, then mutation of the gene should affect the cell's ability to perform the reaction concerned. Likewise, the chief weakness of genetics — inability to distinguish direct from indirect effects — can be tackled biochemically. A geneticist with a newly identified gene can set up assays to determine the enzymatic properties of the protein encoded by the gene. If the gene does what the geneticist thinks it does *in vivo*, then the protein encoded by that gene should have the proper enzymatic activity *in vitro*.

Of course, combining biochemistry and genetics is not always so straightforward. In some experimental animal systems, genetic manipulation techniques are not yet advanced enough to allow specific mutations to be made in a particular gene. Also, there are often difficulties in developing biochemical assays for biological processes that have not been fully defined *in vivo*.

These challenges have been met, at least in part, by molecular biologists, who use recombinant DNA technology to isolate, mutate and analyse genes and their protein products: molecular-biological tools help to bridge the gap between biochemistry and genetics. Indeed, it is now the combination of biochemistry, genetics and molecular biology that drives progress in biology, each providing checks and balances on the others. **D.M.C. & R.A.Y.**

as transcriptional regulators. Mutations in yeast Srb genes, unlike TAF mutations, generate global defects in class II gene expression and affect their ability to respond to regulators *in vivo*. Purified yeast RNA polymerase II holoenzyme can respond to activators *in vitro* in the absence of TAFs.

In this context, it is possible that the TAFs are indeed generally involved in the response to activators *in vivo* but are ancillary to the holoenzyme at most promoters. Activators can interact *in vitro* with components of the holoenzyme as well as TAFs[5,6]. Contact between artificial activators and almost any component of the transcription-initiation apparatus is sufficient to activate transcription *in vivo*[7,8].

Another possibility is that the TAFs function at only a small number of promoters *in vivo*. Struhl and colleagues[4] found two genes whose rate of transcription decreases when expression of some TAFs is shut down *in vivo*. It is intriguing that the promoters of both these genes have TATA boxes whose sequence deviates from the consensus. TAFs are required for transcription from such promoters *in vitro*; furthermore, at least one TAF binds directly to core promoter elements expected to be important for guiding transcription from promoters lacking consensus TATA boxes[1,2].

The observation that inactivation or elimination of TAFs is lethal to cells suggests that TAFs are critical for the expression of certain essential genes. TAFs appear to be involved in cell-cycle progression in yeast and mammalian cells. Green and colleagues[3] point out that inactivation of particular yeast TAFs can cause growth arrest at distinct stages in the cell cycle. One of their temperature-sensitive yeast $TAF_{II}145$ mutants has a phenotype remarkably similar to that found in mammalian cells with a mutation in the homologue $TAF_{II}250$; the mammalian temperature-sensitive cell line arrests growth at the G1 stage of the cell cycle when the temperature is raised, but does not exhibit a global defect in class II gene transcription (ref. 9 and references therein).

Transcription in yeast and mammalian cells almost certainly follows the same fundamental principles, but there is no doubt that mammalian cells need to respond to more complicated and diverse regulatory signals. For example, whereas yeast have only a few developmental programmes, mammals have several hundred. If TAFs are necessary for integrating responses to diverse regulatory controls such as those governing the cell cycle and cell fate, they may be essential for expression of a higher proportion of genes in mammalian cells than in yeast.

The final textbook entry explaining exactly how and where TAFs functio *in vivo* remains to be written. Whatever the outcome, however, the history of gene-activation research underscores the importance of combining genetic an biochemical approaches. □

David M. Chao and Richard A. Young are in the Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, Massachusetts 02142, and the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.

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A mammalian SRB protein associated with an RNA polymerase II holoenzyme

David M. Chao*, Ellen L. Gadbois*, Peter J. Murray*, Stephen F. Anderson†, Michelle S. Sonu*, Jeffrey D. Parvin† & Richard A. Young*

* Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, Massachusetts 02142, and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA
† Division of Molecular Oncology and Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115, USA

A LARGE multisubunit complex containing RNA polymerase II, general transcription factors and SRB regulatory proteins initiates transcription of class II genes in yeast cells[1–4]. The SRB proteins are a hallmark of this RNA polymerase II holoenzyme as they are found only in this complex, where they contribute to the response to regulators[4–8]. We have now isolated a human homologue of the yeast SRB7 gene and used antibodies against human SRB7 protein to purify and characterize a mammalian RNA polymerase II holoenzyme containing the general transcription factors TFIIE and TFIIH. This holoenzyme is more responsive to transcriptional activators than core RNA polymerase II when assayed in the presence of coactivators.

A human complementary DNA clone encoding a protein similar to *Saccharomyces cerevisiae* SRB7 was isolated from a lymphocyte cDNA library by using information derived from expressed sequence tags. The sequence of this human cDNA clone (hSRB7) predicts a 144-amino-acid protein with a relative molecular mass of 15,700 (15.7K) (Fig. 1*a*). The sequence of the predicted protein is 35% identical to yeast SRB7 (ySRB7) (Fig. 1*b*). We tested hSRB7's ability to substitute functionally for ySRB7 by determining whether hSRB7 could complement a complete deletion of the yeast gene. Although full-length hSRB7 failed to complement a ySRB7 deletion, several chimaeras containing the amino terminus of the human protein and the carboxy terminus of the yeast protein were able to do so (Fig. 1*c*, *d*). The complementing chimaera with the largest amount of hSRB7 contains 57% human sequence.

The yeast SRBs interact functionally and physically with the RNA polymerase II C-terminal repeat domain (CTD; reviewed in ref. 4). If the protein encoded by hSRB7 is a genuine homologue of yeast SRB7, then it should be among a small subset of cellular proteins capable of binding to a recombinant CTD column[6]. Extracts were prepared from yeast, HeLa and calf thymus and

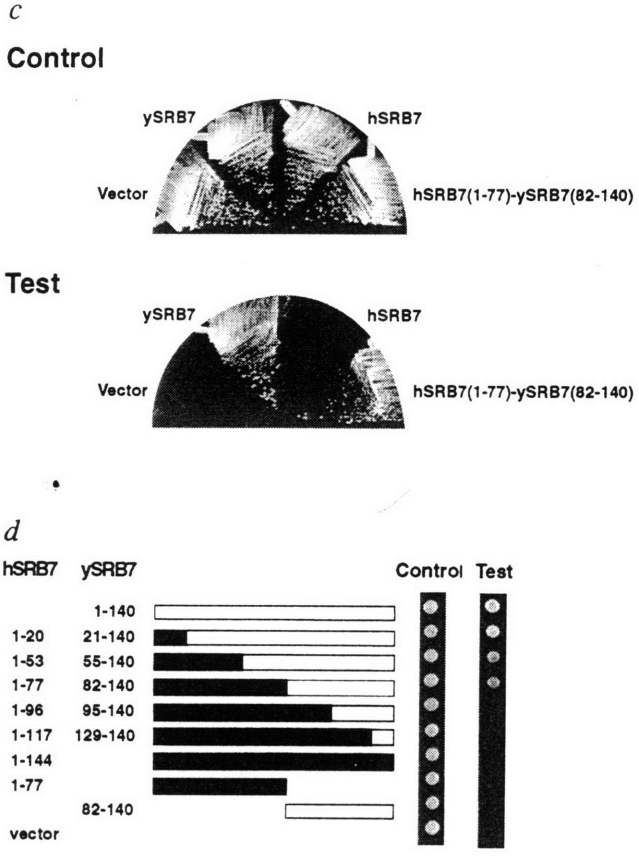

FIG. 1 *a*, Human SRB7 sequence (GenBank accession number u46837). *b*, Human and yeast SRB7 alignment. Vertical lines indicate identity; colons, comparison value ⩾ 0.5; and dots, comparison value ⩾ 0.1 as defined by the program BESTFIT. *c*, Complementation of yeast SRB7 deletion mutant by hSRB7–ySRB7 chimaera. Control: yeast with SRB7 deletion covered by ySRB7 plasmid and the indicated construct. Test: yeast with SRB7 deletion and the indicated construct. *d*, Complementation by hSRB7–ySRB7 chimaeras. Chimaeras are represented by normalized bars showing human SRB7 sequences in black and yeast sequences in white. METHODS. dbEST was screened with XREFdb for expressed sequence tags similar to ySRB7 (ref. 20). A description of the cloning of hSRB7 and the construction of hSRB7–ySRB7 chimaeras is available as Supplementary Information at *Nature*'s web site (http://www.nature.com). ySRB7 and hSRB7 were aligned with BESTFIT (Genetics Computer Group, Inc.). Plasmids containing chimaeras were shuffled into yeast strain Z704 (MATa ura3-52, his3Δ200, leu2-3,112, srb7Δ1 (pCH7: SRB7 URA3 CEN))[21]. A representative clone of each strain was tested by streaking or spotting.

subjected to CTD-affinity chromatography (Fig. 2*a*). Western blots of the column eluates confirmed that yeast SRB7 was retained on a CTD column and showed that mammalian SRB7 from HeLa cells and calf thymus was also retained (Fig. 2*b*).

Because yeast RNA polymerase II holoenzyme can be immunoprecipitated with anti-SRB antibodies, similar experiments were used to investigate whether mammalian SRB7 associated with components of the transcriptional apparatus in crude extracts. Indeed, RNA polymerase II was specifically immunoprecipitated by anti-hSRB7 antibody (Fig. 2*c*, *d*). *In vitro* transcription assays confirmed the presence of RNA polymerase II and revealed the presence of TFIIE and TFIIH activities in the anti-SRB7 immunoprecipitates (Fig. 2*e*). These results are evidence for a mammalian holoenzyme complex containing at least SRB7, RNA polymerase II, TFIIE and TFIIH.

The complex containing mammalian SRB7 and RNA polymerase II was purified over six columns (Fig. 3*a*). As shown by western blotting, RNA polymerase II and SRB7 coeluted precisely from the last three columns of the purification. Analysis of material from the last column revealed that SRB7 and RNA polymerase II coeluted with subunits of TFIIE (p56) and TFIIH (p89) (Fig. 3*b*, *c* and *d*). The number of coeluting polypeptides present in the SRB7–RNA polymerase II complex is consistent with the complex's estimated size of M_r 2,000K, as determined by gel filtration chromatography of crude extracts (data not shown). The preparation appears close to purity as defined by coelution of the same set of proteins over two columns. However, *in vitro* transcription results indicate that TFIIE and TFIIH are substoichiometric (Fig. 4*a*), suggesting that a portion of their activity was lost by dissociation or inactivation during purification.

We next compared the responses of purified mammalian holoenzyme and core RNA polymerase II to the activator Gal4–VP16 and the coactivators HMG2 (refs 9, 10) and PC4 (refs 11, 12). In four independent experiments, the holoenzyme was more strongly inhibited by PC4 and HMG2 in the absence of activator and showed a modestly enhanced response to activator in the presence of these coactivators (Fig. 4*b*). Transcription by core RNA polymerase II was mildly inhibited by PC4 and HMG2 (compare lanes 1 and 3) and was stimulated ~2-fold by activator (compare ratio of upper and lower bands in lanes 3 and 4). Transcription by holoenzyme was more strongly inhibited by PC4 and HMG2 (compare lanes 5 and 7) and was stimulated ~5-fold by the activator (compare ratio of upper and lower bands in lanes 7 and 8). It will be interesting to study the holoenzyme's response to other coactivators and cofactors, such as TBP-associated factors (reviewed in ref. 13), topoisomerase I (ref. 14, Dr1/NC2 (refs 15, 16) and PC2 (ref. 17).

We have shown that hSRB7 shares sequence homology with its yeast counterpart, that hSRB7–ySRB7 chimaeras functionally complement a yeast SRB7 deletion, that hSRB7 is specifically retained by a CTD column and, most important, that hSRB7 associates with a transcriptionally active 2,000K complex containing RNA polymerase II and general transcription factors. We conclude that hSRB7 is a genuine homologue of a yeast SRB gene

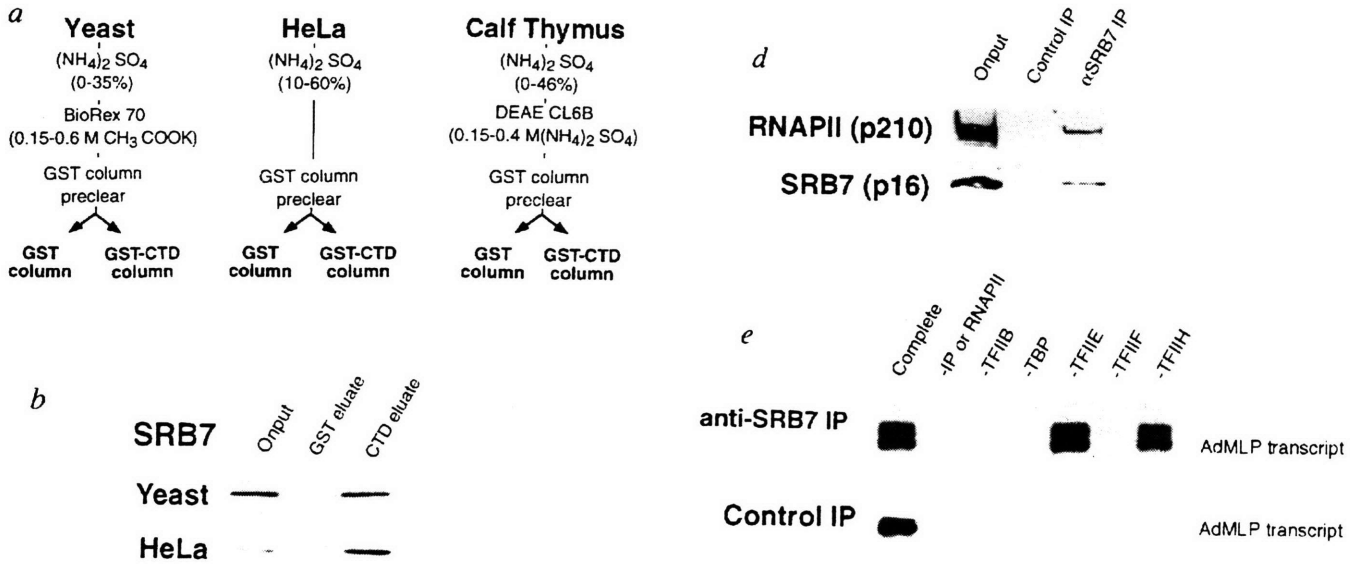

FIG. 2 *a*, CTD-affinity chromatography procedure. *b*, Western blots of CTD-column eluates. Onputs and eluates were probed with anti-ySRB7 or anti-hSRB7. *c*, Immunoprecipitation procedure. *d*, Western blots of anti-hSRB7 immunoprecipitates. Onput and immunoprecipitates were probed with the indicated antibodies. *e*, *In vitro* transcription assays with anti-SRB7 immunoprecipitates. Complete, reaction containing holoenzyme (αSRB7 IP), TBP, TFIIB, TFIIE, TFIIF and TFIIH (top panel), or the same reaction with core polymerase replacing holoenzyme and a control immunoprecipitate (IP) (blocked by peptide) replacing the anti-SRB7 IP (lower panel). In the other lanes, the general factor indicated was omitted from the corresponding complete reaction.

METHODS. Preparation of anti-hSRB7 antibodies, yeast, HeLa and calf thymus extracts, CTD-column eluates and anti-hSRB7 immunoprecipitates is described in Supplementary Information. Monoclonal antibody 8WG16 was used to detect the largest subunit of RNA polymerase II in western blots[22]. *In vitro* transcription was assayed as described[23]. Immunoprecipitates for transcription assay were prepared as for western blotting, except that washes were done with 0.5 ml 60 mM KCl, 50 mM Tris-Cl, pH 7.9, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$.

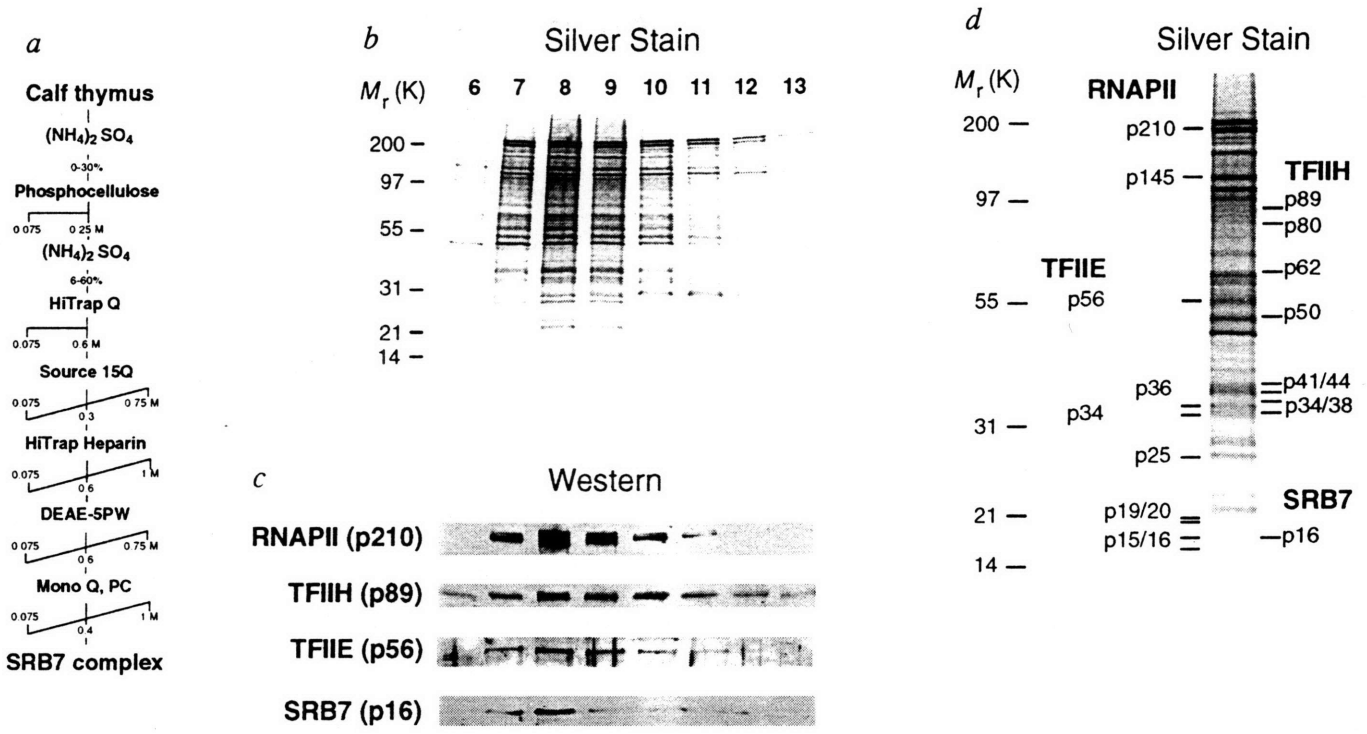

FIG. 3 *a*, Procedure for purifying SRB7, *b* Silver staining of fractions eluted from a Mono-Q column. *c*, Western blotting of fractions eluted from Mono-Q. Fractions were probed with the indicated antibodies. *d*, Proposed identity of holoenzyme polypeptides. Bands that correspond in size to RNA polymerase II, TFIIE and TFIIH subunits and mammalian SRB7 are indicated. METHODS. The purification of mammalian holoenzyme is described in Supplementary Information. Silver staining of purified holoenzyme has been described[6]. For western blotting, rabbit polyclonal anti-TFIIH p89 and anti-TFIIE p56 (gifts from J. Kim, B. Shykind and P. Sharp) were used at a dilution of 1:500, 3 μl of each fraction was analysed. Identities of holoenzyme polypeptides were assigned based on published compositions of core RNA polymerase II[24], TFIIH[25], and TFIIE[26].

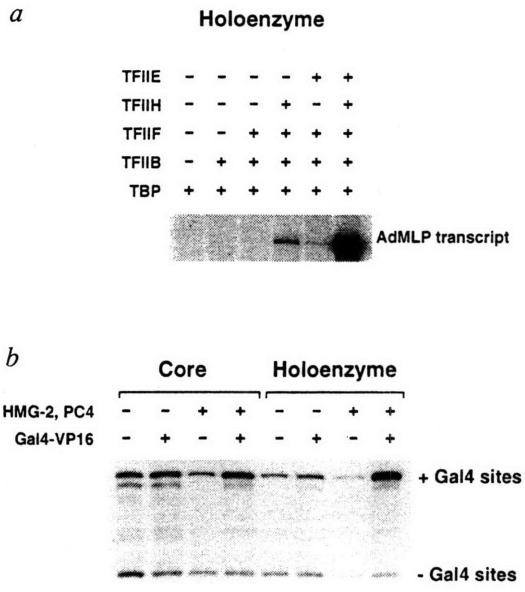

FIG. 4 *a*, *In vitro* transcription assays. Reactions contained column-purified holoenzyme, the general factors indicated, and the adenovirus major late promoter with linear topology. *b*, Response of core RNA polymerase II and column-purified holoenzyme to coactivators and activators. Reactions contained core polymerase or holoenzyme, general transcription factors and/or coactivators and Gal4-VP16. The upper transcript is derived from a template containing the adenovirus major late promoter and three Gal4 binding sites; the lower transcript is derived from a control template containing the same promoter with no Gal4 binding sites.
METHODS. Transcription reactions containing TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH have been described[23]. Holoenzyme was the peak fraction from the Mono-Q column. Protein preparations for all of the basal factors used here have been shown to be free of cross-contamination[27]. HMG2 (30 ng per reaction) and PC4 (50 ng per reaction) were titrated for optimal activation.

and that hRBS7 is a hallmark component of a mammalian RNA polymerase II holoenzyme. We believe that the yeast RNA polymerase II holoenzyme contains RNA polymerase II, SRB proteins and the general factors TFIIB, E, F, and H *in vivo*. Because different holoenzyme purification procedures cause the loss of different subsets of the general transcription factors[1,2,18], the forms of holoenzyme purified so far may be subcomplexes of a larger entity. In this context, it is not yet clear whether the yeast holoenzyme contains TBP *in vivo*. Similarly, it remains to be determined whether the *in vivo* form of the mammalian RNA polymerase II holoenzyme contains all[19] or some of the general transcription factors. The isolation of a human SRB gene and a mammalian RNA polymerase II holoenzyme provides a means for investigating these and other issues in transcriptional regulation and extends the holoenzyme model from yeast to mammals. □

Received 3 November 1995; accepted 19 January 1996.

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ACKNOWLEDGEMENTS. We thank F. Lewitter for assistance with database searches; S. Elledge for a phage library; L. Guarente for a yeast expression plasmid; L. Strasheim and R. Burgess for an unpublished RNA polymerase II purification protocol; J. Kim, B. Shykind and P. Sharp for antibodies and purified transcription factors; and G. Fink, P. Sharp, J. and R. Conaway, D. Reinberg, R. Roeder and U. Schibler for advice and discussion. J.D.P. thanks R. S. Cotran for his support. D.M.C. and E.L.G. are predoctoral fellows of the Howard Hughes Medical Institute. S.F.A. was supported by an institutional training grant from the NIH. This work was supported by NIH grants to R.A.Y.

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 3145–3150, April 1997
Genetics

Functional antagonism between RNA polymerase II holoenzyme and global negative regulator NC2 *in vivo*

ELLEN L. GADBOIS*†, DAVID M. CHAO*†, JOSEPH C. REESE‡, MICHAEL R. GREEN‡, AND RICHARD A. YOUNG*†§

*Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142; †Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139; and ‡Howard Hughes Medical Institute, Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, MA 01605

Communicated by Robert T. Sauer, Massachusetts Institute of Technology, Cambridge, MA, January 15, 1997 (received for review December 11, 1996)

ABSTRACT **Activation of eukaryotic class II gene expression involves the formation of a transcription initiation complex that includes RNA polymerase II, general transcription factors, and SRB components of the holoenzyme. Negative regulators of transcription have been described, but it is not clear whether any are general repressors of class II genes *in vivo*. We reasoned that defects in truly global negative regulators should compensate for deficiencies in SRB4 because SRB4 plays a positive role in holoenzyme function. Genetic experiments reveal that this is indeed the case: a defect in the yeast homologue of the human negative regulator NC2 (Dr1·DRAP1) suppresses a mutation in SRB4. Global defects in mRNA synthesis caused by the defective yeast holoenzyme are alleviated by the NC2 suppressing mutation *in vivo*, indicating that yeast NC2 is a global negative regulator of class II transcription. These results imply that relief from repression at class II promoters is a general feature of gene activation *in vivo*.**

Activation of class II gene transcription in eukaryotes involves the recruitment of a transcription initiation complex that includes the RNA polymerase II holoenzyme (1–6). The yeast RNA polymerase II holoenzyme is a large multisubunit complex containing RNA polymerase II, a subset of the general transcription factors, and SRB regulatory proteins (7–11). Mammalian RNA polymerase II holoenzymes have also been purified, and an SRB7 homologue has been identified as a component of those complexes (12–14).

For some class II genes, regulation appears to involve both positive and negative transcriptional regulators. The negative regulators that have been described include proteins purified for their ability to inhibit transcription *in vitro* (15–21) and genes identified because their products repress transcription from a subset of class II genes *in vivo* (21–29). For example, the human proteins NC1 (15, 16), NC2 or Dr1·DRAP1 (16, 17, 20), and DNA topoisomerase I (18, 19) repress basal transcription *in vitro*. The products of the yeast genes *MOT1* (21–24), *NOT1-4* (25–27), and *SIN4* (28–29) negatively regulate at least a subset of yeast genes *in vivo*. Whether any of these negative regulators are generally employed for class II gene regulation *in vivo* is not yet clear.

The RNA polymerase II C-terminal domain and the associated SRB complex have been implicated in the response to transcriptional activators (7–9, 30, 31). Two holoenzyme components, SRB4 and SRB6, have been shown to play essential and positive roles in transcription at the majority of class II genes in *Saccharomyces cerevisiae* (32). We reasoned that a defect in SRB4 might be alleviated by defects in general negative regulators and that knowledge of such regulators could contribute to our understanding of the mechanisms involved in gene regulation *in vivo*. Here we show that a deficiency in yeast NC2 can compensate for the global transcriptional defects caused by mutations in the SRB4 and SRB6 subunits of the RNA polymerase II holoenzyme and that NC2 is a global negative regulator of class II transcription *in vivo*.

MATERIALS AND METHODS

Genetic Manipulations. Yeast strains and plasmids are listed in Tables 1 and 2, respectively. Details of strain and plasmid constructions are available upon request. Yeast media and manipulation were as described (9). Extragenic suppressors of the temperature-sensitive phenotype of Z628 capable of growth at the restrictive temperature of 36°C were isolated. Dominant and recessive suppressors were identified by mating to Z811 and assaying growth at 36°C on yeast extract/peptone/dextrose (YPD). Complementation groups were established as described (9).

To determine whether the *NCB1* gene is essential for cell viability, the entire coding region was deleted on one of the two chromosomes of a diploid cell, using a single step disruption method (33) and the plasmid RY7136, which carries the deletion allele *ncb1Δ1*. Southern analysis was used to confirm that a single copy of the *NCB1* gene had been deleted. These heterozygous diploid cells were sporulated, and tetrad analysis was performed on YPD plates and scored for growth at a variety of temperatures. Spores with the *ncb1Δ1* allele did not produce colonies, indicating that *NCB1* is essential for cell viability.

DNA Methods. DNA manipulations were performed as described (34). PCR amplifications to produce RY7133, RY7134, RY7136, RY7137, and RY7138 were performed with Vent DNA polymerase (New England Biolabs) as described by the manufacturer. The glutathione *S*-transferase (GST) fusions were constructed as described (13), and the *ncb1Δ1* allele was constructed as described (35).

Cloning and Sequence Analysis. The genomic clone of *NCB1* was isolated by complementation of Z804 with a wild-type genomic library (35). The wild-type gene was further localized by subcloning fragments of the genomic insert and repeating the screen. The clone with the smallest insert, RY7135, was sequenced. The genomic clone of *NCB1* was used to confirm the identity of each member of the complementation group and to identify additional members. RY7138 was created from RY7135 *in vivo* by transforming Z804 with

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Abbreviations: TBP, TATA box-binding protein; GST, glutathione *S*-transferase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. U18917 and U32274).

§To whom reprint requests should be addressed.

Table 1. Yeast strains

Strain	Genotype
Z579	*MATa ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3* [pCT127 (*SRB4 LEU2 CEN*)]
Z628	*MATa ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3* [pCT181 (*srb4-138 LEU2 CEN*)]
Z804	*MATa ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3 ncb1-1* [pCT181 (*srb4-138 LEU2 CEN*)]
Z805	*MATa ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3 ncb1-1* [pCT15 (*SRB4 URA3 CEN*)]
Z806	*MATα ura3-52 his3Δ200 leu2-3,112 ncb1Δ1::HIS3* [RY7135 (*NCB1 URA3 CEN*)]
Z807	*MATα ura3-52 his3Δ200 leu2-3,112 ncb1Δ1::HIS3* [RY7137 (*NCB1* 5′ FLAG tag *LEU2 CEN*)]
Z811	*MATα ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3* [pCT181 (*srb4-138 URA3 CEN*)]

linearized RY7135 lacking *NCB1* coding DNA and then isolating the plasmid from a transformant that had repaired the plasmid with the mutant *ncb1-1* sequence from the chromosome (33). *NCB1* and *ncb1-1* were completely sequenced on each strand using DNA from RY7135 and RY7138, respectively. Double-stranded sequencing with dideoxynucleotides and Sequenase (United States Biochemical) was carried out as described by the manufacturer using T3 and T7 promoter primers and internal oligonucleotide primers. Sequence comparison analysis was performed at the National Center for Biotechnology Information using the BLAST network service (36). The *ncb1-1* mutant allele contained a single base pair deletion at nucleotide 340, causing a frameshift and a translational stop at nucleotides 347–349 (see Fig. 1*b*). Unlike the RY7135 plasmid, RY7138 did not prevent growth at 36°C when transformed into Z804, indicating that the correct gene was cloned.

Antibodies. Recombinant yNC2α and yNC2β proteins were purified for generating polyclonal antibodies in rabbits. Recombinant proteins were derived from *Escherichia coli* containing pGEX-4T-3 (Pharmacia) constructs RY7133 and RY7134 as described (37). The antibodies were used to detect yNC2α and yNC2β in Western blots at a dilution of 1:250 or 1:500.

Purification of Yeast NC2. TATA box-binding protein (TBP) affinity chromatography was performed as described (38) starting with 1.2 kg of cell pellet. Approximately 60% of the total cellular amount of each NC2 subunit was eluted in 1 M KOAc. One-half of the 1 M KOAc eluate (80 ml; 3.3 mg) was dialyzed against buffer T plus 0.003% Nonidet P-40. The dialyzed sample was applied at 1 ml/min to a 1-ml HiTrap SP cartridge (Pharmacia), which was washed with 10 ml of buffer A (20 mM K-Hepes, pH 7.6/1 mM EDTA/10% glycerol and protease inhibitors) plus 100 mM KOAc. Bound proteins were eluted with a 10-ml gradient of buffer A from 100 to 1,000 mM KOAc at 0.25 ml/min. Peak NC2 fractions were pooled, frozen in liquid nitrogen, and stored at −70°C until use. One-half of the peak NC2 fractions (1 ml, 80 μg) was diluted with 2.7 ml of buffer B (20 mM Tris·OAc, pH 7.8/1 mM EDTA/10% glycerol) and applied to a DEAE 5PW 5/5 column (TosoHaas, Montgomeryville, PA) at 0.5 ml/min. The column was washed with 5 ml of buffer B plus 100 mM KOAc, and bound proteins were eluted with a 12-ml gradient of buffer B from 100 to 1,000 mM KOAc. The peak of NC2 contained 50 μg of total protein. SDS/PAGE and silver staining were performed as described (8).

Construction of FLAG-Tagged NC2α Yeast Strain. Plasmid RY7137 was constructed by amplifying the *NCB1* gene (including regulatory sequences) with two sets of overlapping primers to add a FLAG epitope (IBI) to the N terminus of yNC2α. The two PCR products were gel-purified and combined, and the entire FLAG-tagged *NCB1* gene was amplified with primers adding 5′ *Hin*dIII and 3′ *Bam*HI cloning sites. The final PCR product was cloned into plasmid pUN105 (39). RY7137 was transformed into a Z806, a yeast strain containing the *ncb1Δ1* deletion, by plasmid-shuffle techniques (40) to produce Z807. The FLAG-tagged *NCB1* was fully functional and able to complement the *ncb1Δ1* deletion.

Table 2. Plasmids

Plasmid	Description
RY7133	*NCB1* in pGEX-4T-3 (Pharmacia)
RY7134	*NCB2* (amino acids 13–146) in pGEX-4T-3
RY7135	*NCB1* (1.3 kb) *URA3 CEN*
RY7136	*ncb1Δ1::HIS3* in pBluescript II SK(+)
RY7137	*NCB1* 5′ FLAG tag (IBI) in pUN105
RY7138	*ncb1-1* (1.3 kb) *URA3 CEN*

Purification of FLAG-Tagged Yeast NC2 and *in Vitro* Transcription Assays. Yeast strain Z807 was grown in YPD to late log phase and harvested by centrifugation. The cell pellet (500 g) was resuspended in 500 ml of 150 mM KOAc/60 mM K-Hepes, pH 7.6/3 mM EDTA and protease inhibitors. The mixture was poured slowly into a bath of liquid nitrogen, the excess liquid nitrogen was decanted, and the frozen cells were blended for 4 min in a Waring blender. The blended cells were stored at −70°C until use. The frozen mixture was thawed at 55°C and centrifuged at 12,000 rpm for 30 min in a GSA (Sorvall) rotor. One volume (600 ml) of buffer A plus 100 mM KOAc and 300 g of damp-dry BioRex 70 (Bio-Rad) resin were added to the supernatant. After stirring for 2 h, the BioRex 70 resin was washed with 1 liter of buffer A plus 0.1 M KOAc on a Buchner funnel. The washed resin was packed into a 5 cm i.d. column and washed with 0.5 liter of buffer A plus 0.1 M KOAc at a flow rate of 10 ml/min. Bound proteins were eluted with buffer A plus 1 M KOAc. Fractions containing protein (115 ml at 4.1 mg/ml) were pooled, frozen in liquid nitrogen, and stored at −70°C until use. BioRex 70 (32 ml) eluate was thawed and mixed with 160 ml of buffer B plus protease inhibitors. The diluted eluate was centrifuged at 12,000 rpm for 30 min in a GSA rotor. The supernatant was applied to a 2-ml FLAG antibody M2 affinity column (IBI), the column was washed with 100 ml of buffer B plus 150 mM KOAc and 10 ml of buffer B plus 50 mM KOAc, and bound proteins were eluted with buffer B plus 50 mM KOAc plus 50 μM FLAG peptide. The eluate (8 ml) was filtered through a 0.2-μm filter and applied to a Mono Q PC 1.6/5 column (Pharmacia) at a flow rate of 0.1 ml/min, the column was washed with 1 ml buffer B plus 50 mM KOAc plus 1 mM DTT, and bound proteins were eluted with a 2-ml gradient of buffer B plus 1 mM DTT from 50 to 2,000 mM KOAc. SDS/PAGE, silver staining, and Western blot analysis were as described in the Fig. 2 legend. *In vitro* transcription reactions were performed with a yeast CYC1 promoter template as described (41) except that 3′ O-MeGTP was added to 40 μM, T1 RNase was omitted, and ethanol precipitations were performed with 400 instead of 600 μl.

Poly(A)$^+$ Blots and S1 Analyses. Aliquots of cells were removed from culture at the times indicated, total RNA was prepared, and poly(A)$^+$ blots, quantitation, and S1 protection analysis were carried out as described (32).

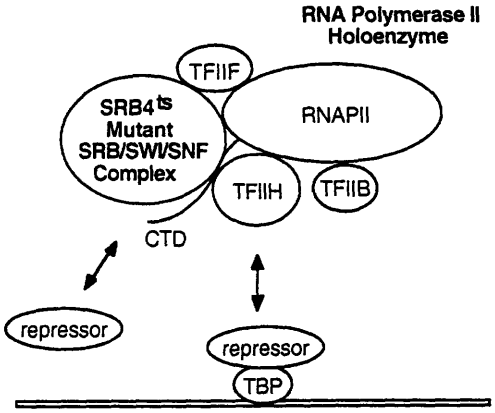
RESULTS

Yeast *ncb1-1* Is an Extragenic Suppressor of the *srb4-138* Mutation. Since SRB4 plays an essential and positive role in class II transcription, we reasoned that a defect in SRB4 might

be alleviated by defects in general negative regulators (Fig. 1*a*). To identify mutations that compensate for a defect in SRB4, 78 spontaneous extragenic suppressors of the temperature-sensitive phenotype of the *srb4-138* allele were isolated. Sixteen of the suppressors were dominant, and 62 were recessive. Five complementation groups were established among the recessive suppressors. One of the recessive suppressing genes was cloned by complementation using a wild-type genomic DNA library and was sequenced. The sequence is identical to the ORF YER159c, which predicts a 142-aa protein with a molecular weight of 15,500 (Fig. 1*b*). A search of the GenBank database (June 7, 1996) revealed that the predicted protein has 39% identity over 99 aa to the NC2α (DRAP1) subunit of human NC2 (Dr1·DRAP1), which binds to TBP and represses transcription *in vitro* (16, 17, 20, 42–44, 47–50). The gene encoding the putative yeast NC2α protein was named *NCB1*. Deletion analysis revealed that *NCB1* is essential for cell viability (data not shown). The mutation present in the suppressing allele, *ncb1-1*, produces a 27-residue C-terminal truncation in the yeast NC2α protein (Fig. 1*b*). Since *NCB1* is an essential gene, the truncation mutation must cause a partial functional defect in the NC2α protein.

Human NC2 consists of two subunits, NC2α and NC2β, both of which are necessary for maximal TBP binding and repression of transcription *in vitro* (42, 43). To determine if there is a yeast homologue of the NC2β subunit, the GenBank database was searched on June 7, 1996 with the human NC2β amino acid sequence. An ORF, D9509.16, that predicts a

a **Genetic Selection for Negative Regulators**



b *NCB1* gene sequence (yNC2α/DRAP1 protein)

```
1    atggcagatcaagtaccagttacaacacaactaccaccaataaaacctgaacatgaggtaccacttgatgctggagggagtccagtaggtaacatgggtaccaactcg
     M  A  D  Q  V  P  V  T  T  Q  L  P  P  I  K  P  E  H  E  V  P  L  D  A  G  G  S  P  V  G  N  M  G  T  N  S

109  aataacaacaacgagctaggtgatgtattcgacagaataaagacacacttccctccggccaaggtaaagaaaataatgcagacagacgaggatataggaaaagtttca
     N  N  N  N  E  L  G  D  V  F  D  R  I  K  T  H  F  P  P  A  K  V  K  K  I  M  Q  T  D  E  D  I  G  K  Y  S
                                                                  ------------------------------
                                                                                helix 1
217  caagccacgcccgtaatagcgggcaggtccctagagttttttatagcgttattggtgaaaaaaagcggggagatggcaagaggacaaggaaccaagagaataaccgcc
     Q  A  T  P  V  I  A  G  R  S  L  E  F  F  I  A  L  L  V  K  K  S  G  E  M  A  R  G  Q  G  T  K  R  I  T  A
     ------------------------------------------------------------------------------------------------------------
                      *     *                 helix 2
325  gaaatactaaaaaaaacaatttaaacgacgaaaaattcgatttcttaagggaaggtctatgcgtagaagaaggccaaacacaaccggaggaagagagtgcctgagca
     E  I  L  K  K  T  I  L  N  D  E  K  F  D  F  L  R  E  G  L  C  V  E  E  G  Q  T  Q  P  E  E  E  S  A  *
        ----------------------------------------------
                        helix 3
```

c *NCB2* gene sequence (yNC2β/Dr1 protein)

```
1    atggctggagactccgataatgtgtcgcttcccaagggtatgttagttatattgttgttgcaaaactcaagctttgatgcgggtactgagcggttatacaaatttaga
     M  A  G  D  S  D  N  V  S  L  P  K  A
                                     ----
109  gaaaacactgaatgatcttagcgaccgtacaaaagatgatatctgaaatactggaccaggatttgatgtttaccaaggatgcaagagaaatcatcatcaacttcggca
                           T  V  Q  K  M  I  S  E  I  L  D  Q  D  L  M  F  T  K  D  A  R  E  I  I  I  N  S  G  I
                        ------------------------------                    ----------------------------
                                        helix 1
217  tagaattcataatgatcctgtcctcgatggcttccgaaatggcggacaacgaggctaagaaaaccatagcgcccgagcacgtgatcaaagcgctagaagagttggagt
       E  F  I  M  I  L  S  S  M  A  S  E  M  A  D  N  E  A  K  K  T  I  A  P  E  H  V  I  K  A  L  E  E  L  E  Y
     ------------------------------------------------------                    --------------------
           helix 2                                                                          helix 3
325  ataatgagtttataccattcttagaggaaatattattgaattttaagggttcccagaaggtgaaagaaactagggattccaagttcaagaagtcaggtcttctcggaag
       N  E  F  I  P  F  L  E  E  I  L  L  N  F  K  G  S  Q  K  V  K  E  T  R  D  S  K  F  K  K  S  G  L  S  E  E

433  aagagctgctacgacaacaagaggagttgtttagacagtcaaggtccagattacaccacaatagtgtatctgatccggttaagtcggaggattcttcttgaatagaag
       E  L  L  R  Q  Q  E  E  L  F  R  Q  S  R  S  R  L  H  H  N  S  V  S  D  P  V  K  S  E  D  S  S  *
```

FIG. 1. Isolation of putative global negative regulators. (*a*) Genetic selection for suppressors of the temperature-sensitive (ts) SRB4 mutant RNA polymerase II holoenzyme. (*b*) Sequence of *NCB1* (ORF YER159c on chromosome V, GenBank accession no. U18917). The suppressing allele, *ncb1-1*, was isolated by gap-repair techniques and sequenced. The suppressing mutation, a single base pair deletion at nucleotide 340, is noted in boldface type. The deletion results in a frameshift causing a translational stop at nucleotides 347–349, also noted in boldface type. Underlined regions indicate homology to α-helices in the histone H2A histone fold (42–44). (*c*) Sequence of *NCB2* (ORF D9509.16 on chromosome IV, GenBank accession no. U32274) with the first and last nucleotide of the intron sequence noted in boldface type. Underlined regions indicate homology to α-helices in the histone H2B histone fold (42–46).

146-aa protein with a molecular weight of 16,700 (Fig. 1*c*) was identified. The predicted protein has 37% identity to human NC2β. The gene, named *NCB2*, contains consensus sequence predicting an intron. Both DNA and cDNA clones containing the coding sequence for *NCB2* were isolated and sequenced, and the intron structure produces a somewhat different amino acid sequence than that predicted by GenBank (Fig. 1*c*). The human NC2 subunits each contain sequences predicting a histone fold structure (42, 43, 51, 52); the yeast NC2 subunits also exhibit this sequence relationship (Fig. 1 *b* and *c*; ref. 44). Interestingly, the C-terminal truncation in the *ncb1-1* suppressing allele removes part of the histone fold in the yeast NC2α subunit (Fig. 1*b*). Deletions in the human NC2 histone folds have been shown to decrease subunit association, TBP binding, and transcriptional repression (42, 43).

Yeast NC2 Binds to TBP and Can Be Purified as a Two-Subunit Complex. If the two yeast gene products are genuine homologues of human NC2, they would be expected to copurify as a complex and to bind to TBP. To determine whether this is the case, a yeast whole-cell extract was subjected to GST and GST–TBP affinity chromatography (Fig. 2*a*). Western blot analyses of the column eluates confirmed that both yeast NC2α and NC2β proteins were specifically retained on the GST–TBP column. The eluate from the GST–TBP column was further purified over two ion-exchange columns. Silver staining and Western blot analyses showed that yeast NC2α and NC2β coeluted over both columns and that the proteins appear to be in equal stoichiometry (Fig. 2*b* and data not shown). Yeast NC2α is not present in a purified RNA polymerase II holoenzyme preparation, so NC2 is unlikely to be a component of the holoenzyme (data not shown). These data confirm that the yeast NC2α and NC2β proteins are stoichiometric subunits of a complex that can bind specifically to TBP.

Highly Purified Yeast NC2 Inhibits Transcription by RNA Polymerase II Holoenzyme *in Vitro*. The observation that a defective form of yeast NC2 can compensate for a weakened RNA polymerase II holoenzyme suggests that yeast NC2 normally functions to repress holoenzyme activity. We tested the ability of purified yeast NC2 to repress transcription by yeast RNA polymerase II holoenzyme *in vitro*. A preparation of yeast NC2 from a strain containing an epitope-tagged NC2α subunit (Fig. 3 *a* and *b*) gave us material of higher yield and purity than from the TBP affinity column. *In vitro* transcription reactions were performed with a yeast CYC1 promoter template, holoenzyme, and fractions from the final column of this yeast NC2 purification (Fig. 3 *c* and *d*). Repression of transcription correlated with the peak of yeast NC2 protein. When an equimolar amount of NC2 was added to RNA polymerase II holoenzyme and TBP, 50% of the maximal inhibition was observed (Fig. 3*d*). The repression of RNA polymerase II holoenzyme transcription by yeast NC2 is consistent with the

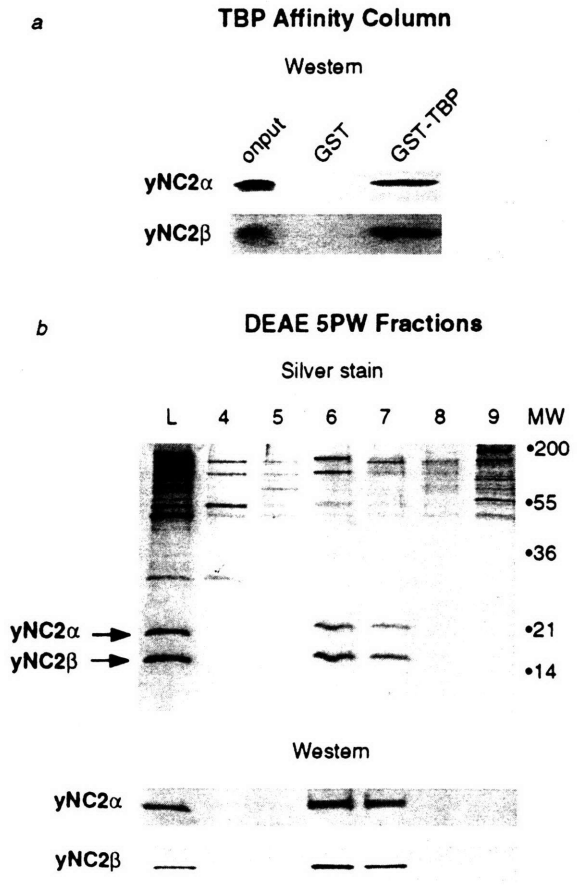


FIG. 2. Yeast NC2 binds to TBP and can be purified as a two-subunit complex. (*a*) Western blot analyses of TBP column onput and eluates with antibodies against yNC2α and yNC2β. Bound proteins were eluted with 2 M KCl. (*b*) Silver-stained SDS/polyacrylamide gel and Western blot analyses of fractions from the final step of the purification (DEAE 5PW).

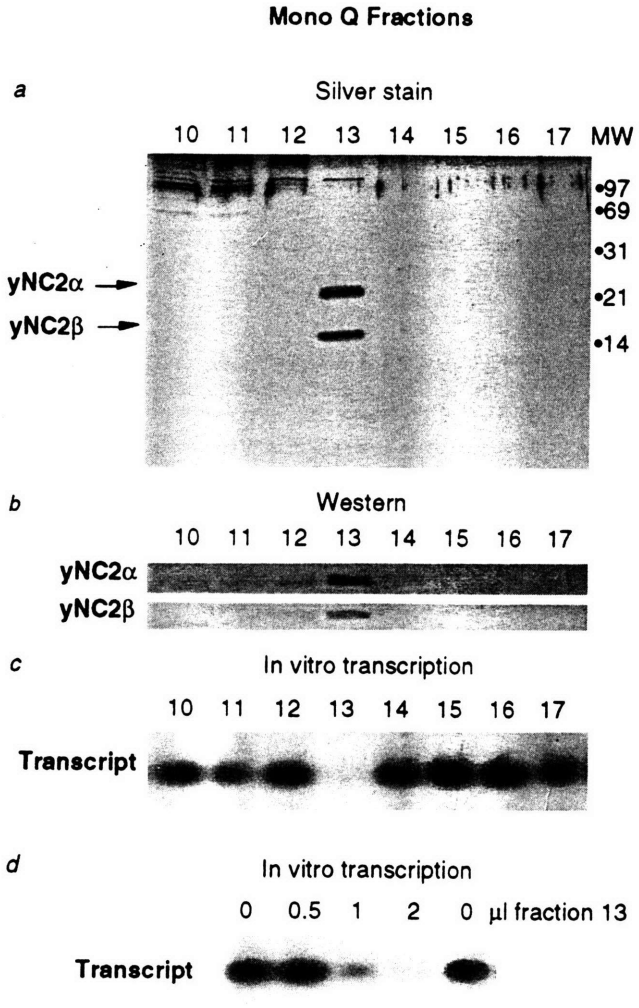


FIG. 3. Highly purified yeast NC2 inhibits transcription by RNA polymerase II holoenzyme *in vitro*. (*a*) Analysis of NC2 Mono Q fractions by SDS/PAGE and silver staining. (*b*) Western blot analyses of Mono Q fractions. (*c*) Influence of Mono Q fractions on *in vitro* transcription by RNA polymerase II holoenzyme. (*d*) Inhibition of *in vitro* transcription by RNA polymerase II holoenzyme with increasing amounts of purified yeast NC2. Assuming a molecular weight of 64,000 for NC2, 0.5 pmol was required for 50% inhibition of an equimolar amount of RNA polymerase II holoenzyme (estimated molecular weight of 2 million) and TBP.

ability of a partial loss-of-function NC2 mutation to suppress a holoenzyme mutation.

NC2 Functions at the Majority of Class II Promoters *in Vivo*. The observation that loss of NC2 function in yeast cells can compensate for a defect in the SRB4 component of the holoenzyme, together with previous evidence that SRB4 functions globally at class II promoters (32), suggests that NC2 may repress transcription at class II promoters in general. To determine whether yeast NC2 functions at the majority of class II promoters *in vivo*, we investigated whether the shutdown of mRNA synthesis observed in cells with the SRB4 temperature-sensitive mutant allele *srb4-138* is reversed by the loss of NC2 function (Fig. 4). Upon shifting cells to the restrictive temperature, the growth rate of the *srb4-138* strain was severely reduced, whereas the *srb4-138 ncb1-1* suppressor strain was only modestly affected (Fig. 4*a*). The levels of poly(A)$^+$ mRNA in these cells were measured immediately before and at several times after the shift to the restrictive temperature (Fig. 4*b*). There was a significant decrease in the mRNA population in the *srb4-138* strain, as observed previously (32). In contrast, there was only a modest decrease in mRNA levels in the *srb4-138 ncb1-1* strain after the temperature shift. Thus, the *ncb1-1* mutation suppresses the general defect in transcription of class II messages caused by the *srb4-138* mutation. Furthermore, the *ncb1-1* mutant in an otherwise wild-type background showed 27% higher levels of poly(A)$^+$ mRNA compared with the wild-type strain under normal conditions (Fig. 4*c*). This result is consistent with the partial loss-of-function of a class II global negative regulator. S1 analysis of individual class II transcripts confirmed that the decline in specific mRNAs in the *srb4-138* strain is reversed in the *srb4-138 ncb1-1* strain (Fig. 4*d*). These results, together with previous evidence that NC2 functions as a repressor of multiple promoters tested *in vitro*, argue that NC2 is a general negative regulator of class II gene transcription.

DISCUSSION

Our results indicate that NC2 is an essential and conserved negative regulator of class II gene transcription. These results extend the known negative regulatory effects of NC2 on core RNA polymerase II *in vitro* (16, 17, 20, 42–44, 47–50) and confirm that this regulator can inhibit transcription by RNA polymerase II *in vivo*.

Since SRB4 has an essential and positive role in transcription at the majority of class II genes in yeast (32), we reasoned that suppressors of a temperature-sensitive SRB4 mutant should include negative regulators. In principle, such regulators could repress most class II genes or they could repress SRB4 specifically. Several lines of evidence argue that NC2 acts globally as a repressor of most, if not all, class II genes. NC2 can repress transcription *in vitro* from a wide variety of mammalian, viral, and yeast promoters (16, 20, 42–44, 50). The NC2 suppressor mutation that compensates for reduced class II gene transcription *in vivo* due to loss of SRB4 also compensates for the global defect due to loss of SRB6 (data not shown). Loss of function of NC2 in otherwise wild-type cells results in increased levels of poly(A)$^+$ mRNA. These data do not prove that NC2 regulates all class II genes, but they are most consistent with a global role for this repressor.

Mechanism of Yeast NC2 Repression. Much is already known about the biochemistry of NC2 repression; NC2 binds to TBP on promoter DNA and subsequently inhibits the binding of TFIIA and TFIIB *in vitro* (16, 17, 42, 43, 50). NC2 binds to the same basic region of TBP as TFIIA (50), suggesting that NC2 physically blocks TFIIA from binding to TBP.

The other proteins known to have global negative regulatory properties are the histones (53, 54), which share notable structural features with NC2/Dr1·DRAP1. The presence of the histone fold motif in NC2 raises the intriguing possibility

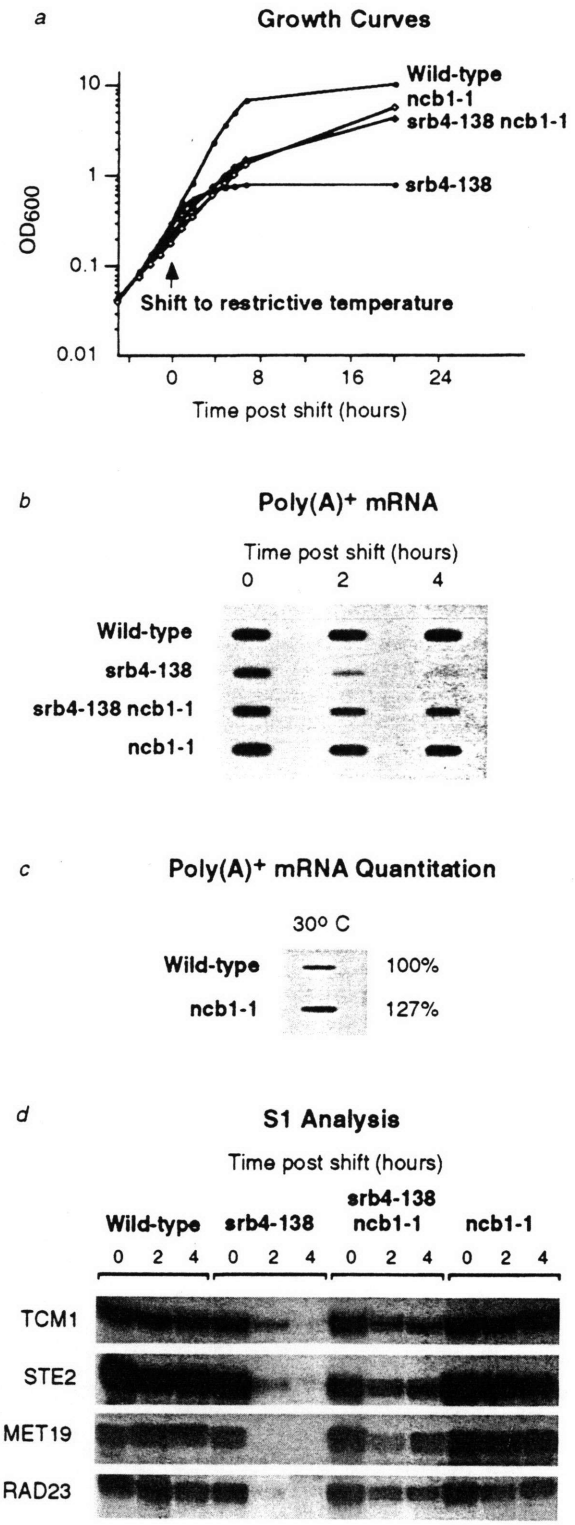


FIG. 4. Loss of yeast NC2 function compensates for the global defect in class II gene expression caused by the SRB4 mutant holoenzyme. (*a*) *ncb1-1* mutation suppresses the growth defect of the *srb4-138* mutant strain at the restrictive temperature. Growth of wild-type (Z579), *srb4-138* (Z628), *srb4-138 ncb1-1* (Z804), and *ncb1-1* (Z805) strains in YPD medium at 30°C and after shifting to the restrictive temperature of 35.5°C. (*b*) The global decline in mRNA levels at the restrictive temperature in *srb4-138* mutant strain is alleviated by the *ncb1-1* mutation. (*c*) Global levels of mRNA are increased in the *ncb1-1* strain relative to the wild-type strain. (*d*) The decrease in synthesis of individual class II messages at the restrictive temperature in the *srb4-138* mutant strain is reversed by the *ncb1-1* mutation.

of interactions with other histone fold-containing proteins. Histones H2A, H2B, H3, and H4 all contain histone folds (51),

as do the yeast HAP3 and HAP5 activator proteins (CBF proteins in mammals; ref. 52) and several TAF_{II}s (45, 46). These TAF_{II}s and histones are able to interact with each other *in vitro* through their histone fold regions (45). Thus, NC2 might introduce a nucleosome-like structure at the promoter, either by itself or with other histone fold-containing proteins.

Transcription Activation and Relief from Repression. The SRB components of the RNA polymerase II holoenzyme contribute to the response to transcriptional activators (7–9). We have shown that a partial loss in NC2 function compensates for deficiencies in SRB4 and SRB6 functions. These results indicate that relief from NC2 inhibition is a required step during transcription initiation at most class II promoters *in vivo*. Evidence consistent with this view has recently emerged from a study of *SUC2* gene regulation. Prelich and Winston (55) isolated yeast mutations that suppress a deletion of the upstream activating sequence in the *SUC2* promoter. The mutant genes that compensated for the absence of *SUC2* activator function included several histones, certain *SPT* genes, and other unidentified genes called *BUR* genes (*B*ypass *U*AS *R*equirement). The *bur6* mutant allele was recently found to be a partial loss-of-function mutation in *NCB1* (56). The observation that *BUR6* is identical to *NCB1* indicates that a loss in NC2 function can compensate for the loss of an activator. These data support the model that activators function to recruit the transcription apparatus, which must overcome negative regulation by NC2 to initiate transcription.

We thank R. Roeder, P. Sharp, C. Thompson, A. Hoffmann, T. Lee, H. Madhani, and S. Liao for advice and discussions; J. Madison and E. Shuster for strains and plasmids; L. Ziaugra, E. Jennings, and V. Tung for technical assistance; F. Lewitter for assistance with database searches; and G. Prelich, A. Goppelt, M. Meisterernst, J. Kim, and D. Reinberg for sharing data prior to publication. E.L.G. and D.M.C. are predoctoral fellows of the Howard Hughes Medical Institute. J.C.R. is a postdoctoral fellow of the Damon Runyon-Walter Winchell Cancer Research Foundation. This work was supported by National Institutes of Health grants to R.A.Y.

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Association of an activator with an RNA polymerase II holoenzyme

Christoph J. Hengartner, Craig M. Thompson, Jianhua Zhang, David M. Chao, Sha-Mei Liao, Anthony J. Koleske, Sara Okamura, and Richard A. Young

Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142 USA and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 USA

RNA polymerase II holoenzymes have been described that consist of RNA polymerase II, a subset of general transcription factors, and four SRB proteins. The SRB proteins, which were identified through a selection for genes involved in transcription initiation by RNA polymerase II in vivo, are a hallmark of the holoenzyme. We report here the isolation and characterization of additional *SRB* genes. We show that the products of all nine *SRB* genes identified thus far are components of the RNA polymerase II holoenzyme and are associated with a holoenzyme subcomplex termed the mediator of activation. The holoenzyme is capable of responding to a transcriptional activator, suggesting a model in which activators function, in part, through direct interactions with the holoenzyme. Immunoprecipitation experiments with anti-SRB5 antibodies demonstrate that the acidic activating domain of VP16 specifically binds to the holoenzyme. Furthermore, the holoenzyme and the mediator subcomplex bind to a VP16 affinity column. These results provide a more complete description of the RNA polymerase II holoenzyme and suggest that this form of the transcription apparatus can be recruited to promoters via direct interactions with activators.

[*Key Words*: RNA polymerase II; holoenzyme; carboxy-terminal domain; genetic suppressors; transcription initiation; SRBs; transcription activation]

Received December 13, 1994; revised version accepted March 14, 1995.

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

Selective transcription initiation in vitro by the 12-subunit core RNA polymerase II was shown previously to require the action of at least five general initiation factors: TATA-binding protein (TBP), TFIIB, TFIIE, TFIIF, and TFIIH (for review, see Conaway and Conaway 1993; Zawel and Reinberg 1993). Consistent with these data, selective transcription initiation in vitro with the larger form of RNA polymerase II holoenzyme required TBP and TFIIE (Koleske and Young 1994), and initiation with the smaller form required TBP, TFIIB, TFIIE, and TFIIH (Kim et al. 1994).

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

The RNA polymerase II holoenzymes are responsive to activators (Kim et al. 1994; Koleske and Young 1994), a feature not observed with purified RNA polymerase II and general transcription factors alone (Flanagan et al.

1991, 1992). Thus, these holoenzymes contain components necessary and sufficient for some level of response to transcriptional activators. A subcomplex, called the mediator of activation, can be dissociated from the RNA polymerase II holoenzyme by using monoclonal anti-CTD antibodies (Kim et al. 1994). The purified mediator contains SRB2, SRB4, SRB5, SRB6, SUG1, GAL11, TFIIF, and as yet unidentified polypeptides and is capable of reconstituting the ability of purified RNA polymerase II and general factors to respond to transcriptional activators. Genetic studies suggested previously that GAL11, SUG1, and the SRB proteins are involved in transcriptional regulation (Suzuki et al. 1988; Fassler and Winston 1989; Himmelfarb et al. 1990; Nishizawa et al. 1990; Vallier and Carlson 1991; Swaffield et al. 1992; Yu and Fassler 1993).

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

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

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

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

Results

Extragenic suppressors of RNA polymerase II CTD truncation mutations

Extragenic suppressors of a *S. cerevisiae* RNA polymerase II CTD truncation mutant were isolated to identify components of the transcription apparatus involved in initiation. The cold-sensitive phenotype of *rpb1Δ104* cells containing RNA polymerase II CTDs with 11 intact heptapeptide repeats was used to obtain 75 independent suppressing isolates. Genetic analysis revealed that mutations in a total of nine *SRB* genes suppress growth defects of cells containing a truncated CTD (Table 1). Previously, we described the isolation and characterization of *SRB2*, *SRB4*, *SRB5*, *SRB6*, *SRB10*, and *SRB11* (Nonet and Young 1989; Koleske et al. 1992; Thompson et al. 1993; Liao et al. 1995). Thorough genetic analysis of the 75 independent suppressing isolates led to the identification of three additional genes: *SRB7*, *SRB8*, and *SRB9*.

Table 1. SRB *gene summary*

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

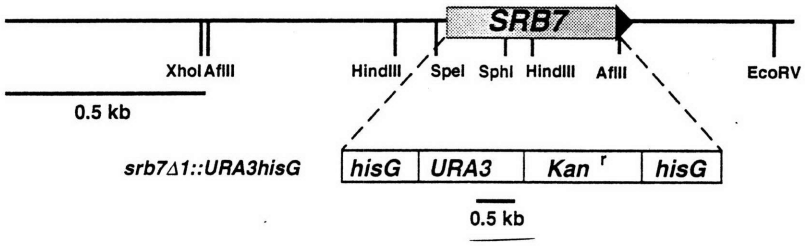
[a](1) Nonet and Young (1989); (2) Koleske et al. (1992); (3) Thompson et al. (1993); (4) Koleske and Young (1994); (5) this paper; (6) Liao et al. (1995).

The suppressing alleles of *SRB2*, *SRB4*, *SRB5*, *SRB6*, *SRB10*, and *SRB11* were found to suppress the conditional phenotypes associated with the CTD truncation mutation *rpb1Δ104* but did not suppress similar conditional phenotypes caused by other mutations in *RPB1* (Nonet and Young 1989; Koleske et al. 1992; Thompson et al. 1993; Liao et al. 1995). Similarly, the mutations *srb7-1*, *srb8-1*, and *srb9-1* were also specific in suppressing conditional phenotypes attributable to CTD truncation mutations (not shown). This specificity of suppression suggests that the *SRB* gene products and the CTD are involved in the same process in transcription initiation.

Genomic DNA clones containing *SRB7*, *SRB8*, and *SRB9* were isolated by genetic complementation. Sequence analysis revealed that *SRB7*, *SRB8*, and *SRB9* are all newly identified genes. The predicted SRB7 protein is 140 amino acids long and has a molecular mass of 16 kD (Fig. 1). Physical mapping showed that *SRB7* is located on the right arm of chromosome IV, ~45 kb distal to *GCN2* (λ clone 6118). Partial sequence analysis of the *SRB8*-coding sequence revealed that it is identical to open reading frame (ORF) YCR81W on chromosome III (Oliver et al. 1992). The SRB8 protein is predicted to be 1226 amino acids in length with a molecular mass of 144 kD (Fig. 2). The predicted SRB9 protein is 1420 amino acids long and has a molecular mass of 160 kD (Fig. 3). *SRB9* maps to the right arm of chromosome IV, ~35 kb centromere distal to *ADE8* (λ clone 5513). A search of the sequence data banks revealed that SRB7, SRB8, and SRB9 do not have significant sequence similarity to previously identified proteins.

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

A



B

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-573  AAAAAAAAAAAATTCAAAGAATCAGCTTATAAAACATATTCAAGGACCATCTGAAGTATCATTCATTCGTTTTTT
-498  ACTCGTTAATCTCATTCATTCGTTTCCTCATTCTTTTTTCTTTGTTCTTTATTTCGGCTATTTTTTCACTATTAA
-423  AATAACTAGAGCTAACAATATTATTTCTTCTGCTTTAGTTACAAAACAAGGACATTCATTTAACTTGGCGTTATC
-348  CCATACATTCGTTTATTATATCTTCTTTTAAAACACAATTTCTTTTACAGTTAAACTTTTCTGATTTATTATATA
-273  TTACTTAAGATTGTTCATATAACTAACATTTATATGCTTATATGCGTGAAGTGCGCTTTTGTAGAACATGTGGCT
-198  GTTTCTGTAGAAGCCTTGTCTTTCTCTGTAATCCTTTAAAGGCCAACCGTACGTGCTTAATTACAAGCTTTGTTC
-123  GCATTGCAAGAAAGTTAGAAAAAAAAATCAATTCTGAAAGATAATTATAATTCAAACGGTAAACCATTGGTTAAAA
 -48  GAGGGACATAACATTTCACTAGTTCAATACATTATATGCTCTTTAACAATGACAGATAGATTAACACAATTGCAG
                                                    M  T  D  R  L  T  Q  L  Q
                                    A(srb7-1)
  28  ATATGTTTAGACCAAATGACGGAGCAATTCTGTGCTACTTTAAACTACATAGATAAGAACCATGGTTTTGAACGA
       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  TTGACCGTAAATGAACCTCAGATGTCCGATAAGCATGCCACAGTAGTACCTCCTGAGGAATTTTCTAACACGATA
       L  T  V  N  E  P  Q  M  S  D  K  H  A  T  V  V  P  P  E  E  F  S  N  T  I

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

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

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

 403  AACTCAAAAAAGAGCACATAAACTTAAGTTTTACAAAGAAATTTGCGAACAGAGGACAGAAAATGTACTATAGTT
       N  S  K  K  S  T  *

 478  ATATGGCAGAGTTAAGCGTATGTATGTTATTCTTATAAATAATTGTGCTACTCTATTTGTACCGGAGAATTATTG
 553  AAGCAATGGGAGAAAAATCATAATGGAGAAAATCTTTCTACGAGTTACTTTGCAAGGCAATCTAACGATTCTAAA
 628  AGACACAATACACTAAAGAAAAAACTTTGGAAGTACAGTTTTTTCCCCAAGTTGAAGTGTGGACTCATTGTGAAG
 703  ATGTAAAAATGTAAAAACCAACCGACAATGCACTCCCAGCCAAATTCATTGTAGACCTCCCATTTGATAGAAAAG
 778  GAAGGTTCAGCAGTTGTCCACGGATTCCAAGATATCATTCTCTTACATTGCACGCACATGAAAATGATC  846
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Figure 1. Map and sequence of the *SRB7* gene. (*A*) Restriction map of a 2.0-kb DNA fragment from pCH7 containing the *SRB7* gene. The entire coding region of *SRB7* was replaced with a 5.5-kb DNA fragment containing the *URA3* and kanamycin genes flanked by direct repeats of *Salmonella hisG* DNA to create the deletion allele *srb7Δ1::URA3hisG*. (*B*) Sequence of the *SRB7* gene and adjacent DNA. The predicted 140-amino-acid sequence of the SRB7 protein is shown below the sequence of the gene. Positive numbering of the nucleotides begins with the predicted start site of translation. The *srb7-1* mutation is a G → A transition (nucleotide 61) that changes amino acid 21 from Ala to Thr.

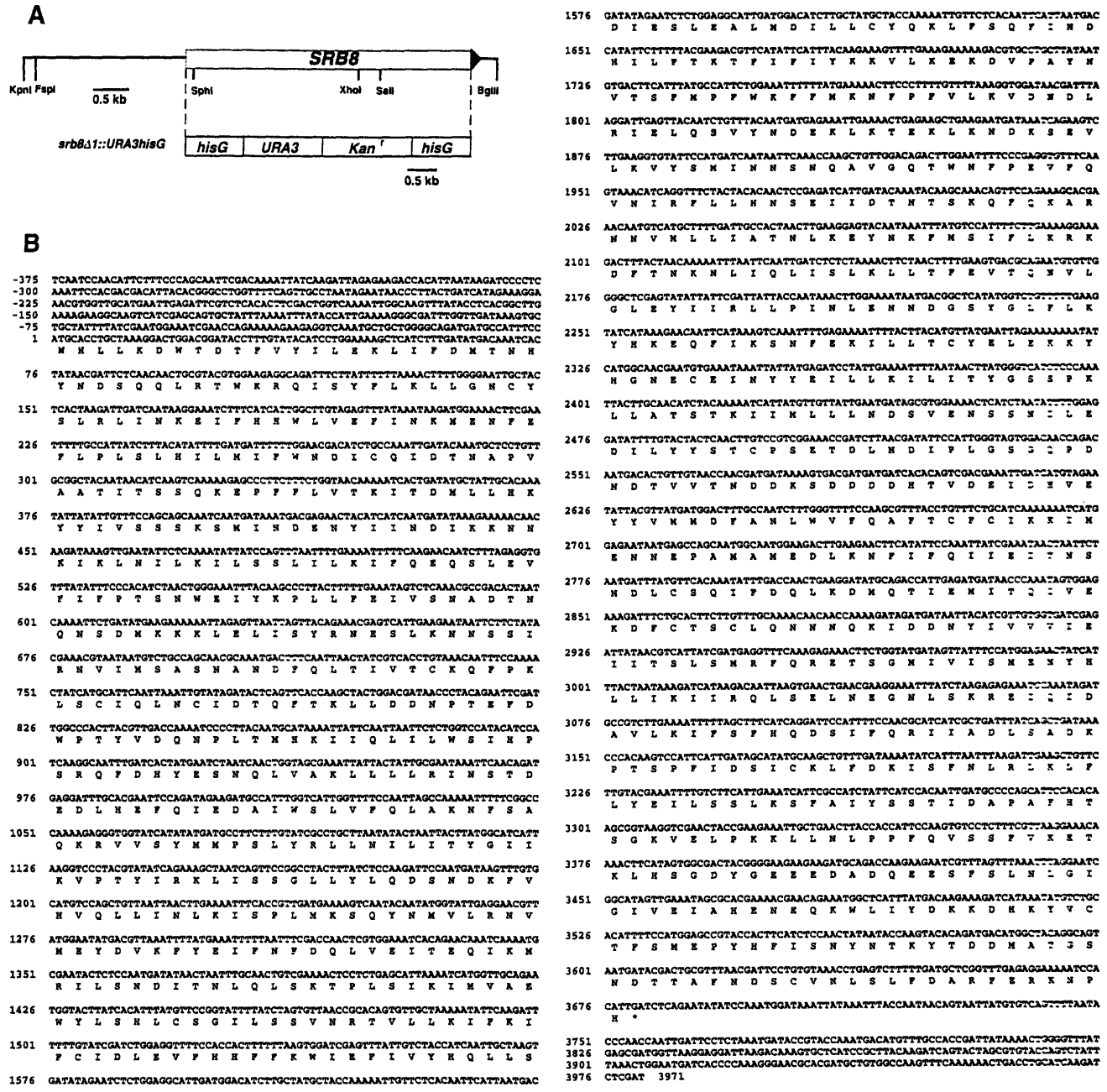
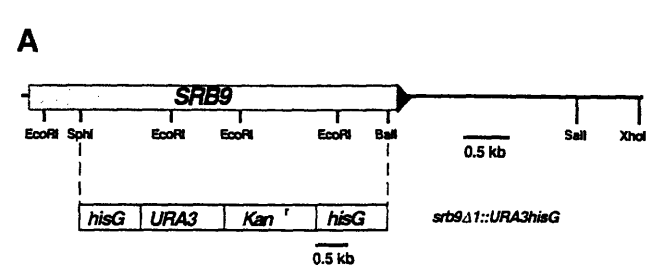


Figure 2. Map and sequence of the *SRB8* gene. (*A*) Restriction map of a 6.0-kb DNA fragment from pSL311 containing the *SRB8* gene. The entire coding region of *SRB8* was replaced with a 5.5-kb DNA fragment containing the *URA3* and kanamycin genes flanked by direct repeats of *Salmonella hisG* DNA to create the deletion allele *srb8Δ 1::URA3hisG*. (*B*) Sequence of the *SRB8* DNA and adjacent DNA. The predicted 1226-amino-acid sequence of the SRB8 protein is shown below the sequence of the gene. Positive numbering of the nucleotides begins with the predicted start site of translation. The *SRB8*-coding sequence is identical to ORF YCR81W (Oliver et al. 1992).

All nine SRBs are components of an RNA polymerase II holoenzyme

An RNA polymerase II holoenzyme has been purified and shown to consist of RNA polymerase II, the general transcription factors TFIIB, TFIIF, and TFIIH and the SRB proteins SRB2, SRB4, SRB5, SRB6, SRB10, and SRB11 (Thompson et al. 1993; Koleske and Young 1994; Liao et al. 1995). We investigated whether SRB7, SRB8, and SRB9 are also components of this holoenzyme. Rabbit polyclonal antibodies were generated against recombinant SRB7, SRB8, and SRB9. Column fractions from the final purification step of the RNA polymerase II holoenzyme were tested in reconstituted transcription reactions and subjected to Western blot analysis with antisera specific to RNA polymerase II and SRB proteins (Fig. 4A). SRB7, SRB8, and SRB9 coeluted with the other SRB proteins, RNA polymerase II, and transcriptional activ-



B

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 -72 TACTTTGTTGAAGTAAGGTGTAACTATATTTTAAGAACGTTTAAGCTGGATATCAAGATCTGAGGAGGTAGTATG
                                                                             M
   4 AGTTCTGACGCTTCCACGTACAGACTTGAGGATGTTTTATCCAGCTTCTATAGAGTGGAGAAAATCAAAAAGATC
       S  S  D  A  S  T  Y  R  L  E  D  V  L  S  S  F  Y  R  V  E  K  I  K  K  I
  79 AACTATCATCAGTACATTTCTAAAGCCCAAAACGATCAATGGTCTATCCAAATGGAATTCATGCTACGGAAGCAG
       N  Y  H  Q  Y  I  S  K  A  Q  N  D  Q  W  S  I  Q  M  E  F  M  L  R  K  Q
 154 GATCCAAAGACTCTAGTTGCGCTGCTTTCAAGGGATTTATGGTGTTTCAGTATAAATGATGATCCGGTACCGACA
       D  P  K  T  L  V  A  L  L  S  R  D  L  W  C  F  S  I  N  D  D  P  V  P  T
 229 CCTCCTGCGATAGAACATAAACCAGTGAGCCCAGATAAAATCGGAACTTTCACTGCCGATTATTCAAAGCCAAAC
       P  P  A  I  E  H  K  P  V  S  P  D  K  I  G  T  F  T  A  D  Y  S  K  P  N
 304 TTACCGCCACACTATGCTCTTTTTTTAAAAGCTTTAAGAAGGAAAATTTACATTAATTTGGCATTAGGTTCACAC
       L  P  P  H  Y  A  L  F  L  K  A  L  R  R  K  I  Y  I  N  L  A  L  G  S  H
 379 AATAAGCTAATACAATTTGGGAATGCCTGCATATCATTAAGCGGAGTGCCAAATTATCTCGTACAGCTAGAACCA
       N  K  L  I  Q  F  G  N  A  C  I  S  L  S  G  V  P  N  Y  L  V  Q  L  E  P
 454 CACCTTTTTGTAAACGGAGATCTCACAGTGTCGTTATGTGCCAAGAACATGGGATTAGTACCAATGAAGGAGGAA
       H  L  F  V  N  G  D  L  T  V  S  L  C  A  K  N  M  G  L  V  P  M  K  E  E
 529 AATTTGGAAGAATCTTTCCTTTCAAAGCATGCGCTTTATTTAGCACCATCTGGAATAAGGATGCATTTGGCCCCT
       N  L  E  E  S  F  L  S  K  H  A  L  Y  L  A  P  S  G  I  R  M  H  L  A  P
 604 GCTTCCAAGCAAGGATACTTGATAACGCCACCAAAACATACAGAACTTCTCTTGACGACGTTAAGTGTATCTCAT
       A  S  K  Q  G  Y  L  I  T  P  P  K  H  T  E  L  L  L  T  T  L  S  V  S  H
 679 GGTATAAACTTACAAAATAAAAAAAATTTGAAATGGGTTGCTGTTGTTCCTGACTTAGGACATCTCAACGGCCAC
       G  I  N  L  Q  N  K  K  N  L  K  W  V  A  V  V  P  D  L  G  H  L  N  G  H
 754 ACACCTACTATAGCTTCGTATTTAACTCCCTTACTTGAAGCAAAGAAGCTAGTATGGCCGCTGCATTTAATCTTC
       T  P  T  I  A  S  Y  L  T  P  L  L  E  A  K  K  L  V  W  P  L  H  L  I  F
 829 GCCCAACCAGTAGCTGATATAGAAAATTCTACTTCCGGAGATCCATCAGAATTTCACTGTTTGCAAGATGCTCTG
       A  Q  P  V  A  D  I  E  N  S  T  S  G  D  P  S  E  F  H  C  L  Q  D  A  L
 904 GATGCCATTGATGATTTCATACAATTAAAGCAAACAGCTGCCTATAGGACTCCAGGAAGTTCCGGCGTATTGAGC
       D  A  I  D  D  F  I  Q  L  K  Q  T  A  A  Y  R  T  P  G  S  S  G  V  L  S
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       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
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       S  I  S  S  Q  P  A  S  Y  H  S  V  Q  E  T  N  K  I  S  P  K  D  F  S  P
1129 AATTTCACAGGCATTGATAAATTAATGCTGTCGCCCAGCGATCAATTTGCTCCAGCTTTCTTAAATACCCCTAAT
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3154 GTGATCAAGAACGAAGTTTGTGCCAAGATGCCTAACATTGAACTATATTTGAAAGTTATTCCTATGGATTTCATT
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4204 ACTTCCGCCGCTACTGTGCATACCGCTACTTCTTCATCAATTATTCTCTCGGATAAATAAACTTTTTCCGGCAAC
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4654 AAGTTCTCTTGATTAGCAAGTAGTTCTTACATCGCAGGAATCTTATGTT 4702
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Figure 3. Map and sequence of the *SRB9* gene. (*A*) Restriction map of a 7.3-kb DNA fragment from pCH47 containing the *SRB9* gene. Most of the coding region of *SRB9* was replaced with a 5.5-kb DNA fragment containing the *URA3* and kanamycin genes flanked by direct repeats of *Salmonella hisG* DNA to create the deletion allele *srb9Δ1::URA3hisG*. (*B*) Sequence of the *SRB9* DNA and adjacent DNA. The predicted 1420-amino-acid sequence of the SRB9 protein is shown below the sequence of the gene. Positive numbering of the nucleotides begins with the predicted start site of translation. A sequence encoding a polyglutamine repeat, a motif found in several transcription factors (e.g., Sp1, $dTAF_{II}110$, OCT2), is underlined (Clerc et al. 1988; Courey and Tjian 1988; Hoey et al. 1993).

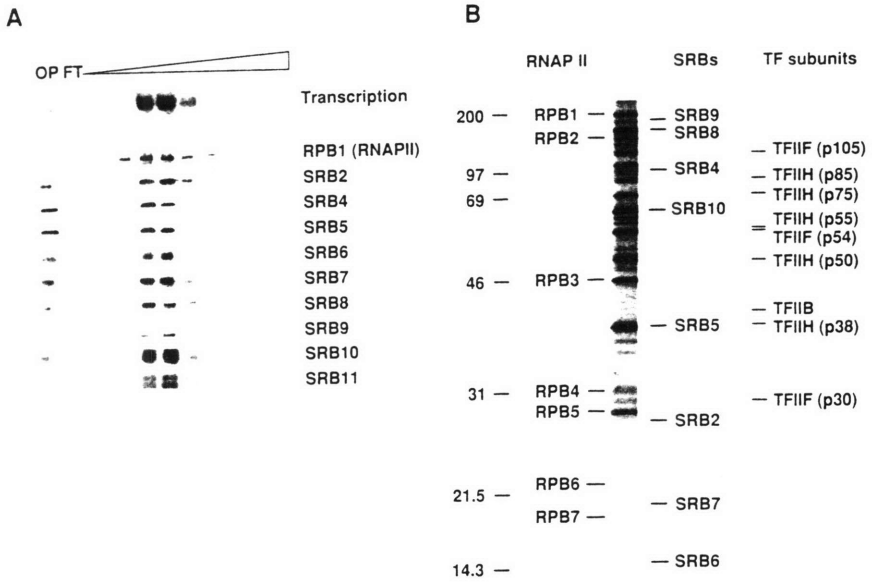

Figure 4. All SRBs are components of an RNA polymerase II holoenzyme. (*A*) RNA polymerase II holoenzyme was purified as described (Koleske and Young 1994). Holoenzyme loaded onto a Mono S column, the last chromatographic step in the purification procedure, was eluted with a 0.1–1.0 M gradient of potassium acetate. The onput (OP) and flowthrough (FT) and a portion of every other fraction eluting between 0.1 and 0.9 M potassium acetate were analyzed for holoenzyme activity (*top*). These samples were also analyzed by Western blot for the presence of RNA polymerase II and SRB proteins. The Western blot for SRB11 was done with an RNA polymerase II holoenzyme purified independently from cells with an epitope-tagged SRB11 protein; the purification and transcriptional properties of this holoenzyme were identical to the holoenzyme lacking the epitope tag (Liao et al. 1995). (*B*) Polypeptide composition of RNA polymerase II holoenzyme. One microgram of purified holoenzyme was subjected to SDS-PAGE and stained with silver. Proteins in the holoenzyme preparation that correspond in size to subunits of RNA polymerase II, SRB proteins, and general transcription factors are indicated. The numbers used for the subunits of transcription factors TFIIF and TFIIH are from previous reports (Henry et al. 1992; Feaver et al. 1993). The sizes of protein molecular mass standards are indicated in kD. This and Figs. 5–8 were prepared from digital replicas of primary data scanned using a UMAX UC840 Max Vision digital scanner.

ity. Thus, all nine SRB genes identified through our genetic selection encode components of the RNA polymerase II holoenzyme.

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

SRB proteins are components of the mediator of activation

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

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

Acidic activator VP16 coimmunoprecipitates with RNA polymerase II holoenzyme

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

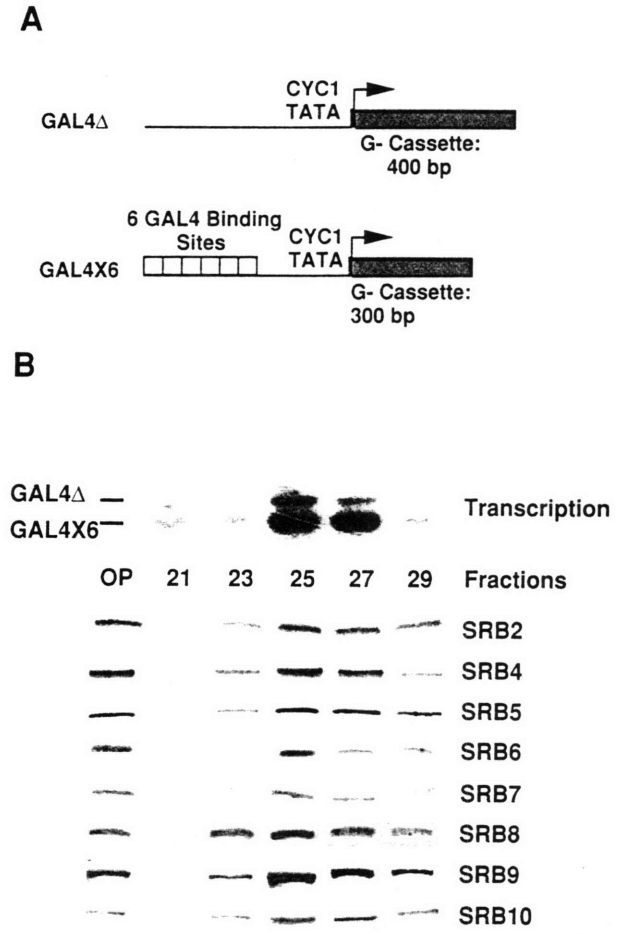

Figure 5. SRBs are components of the mediator of activation. (*A*) In vitro transcription templates. (*B*) A mediator preparation was loaded onto a MonoQ column, the last column in the purification procedure, and eluted with a 0.4–0.8 M gradient of potassium acetate. Mediator activity peaked in fraction 25 (*top*). This and nearby fractions were analyzed by Western blot for the presence of SRB proteins.

activation domain (Lin et al. 1991; Goodrich et al. 1993; Xiao et al. 1994). To investigate whether the holoenzyme can bind to the acidic activation domain of the activator VP16, we immunoprecipitated the RNA polymerase II holoenzyme from a crude yeast protein preparation in the presence of wild-type and mutant VP16 activator proteins. The HSV *trans*-activator VP16 has been characterized extensively, and analysis of VP16 mutants suggests the presence of two activation subdomains within the highly acidic carboxy-terminal 78 amino acids (Triezenberg et al. 1988; Regier et al. 1993). The VP16$^{\Delta 456FP442}$ mutant is defective in transcriptional activation; one subdomain of transcriptional activation is deleted (amino acids 457–490), and the second subdomain (amino acids 413–456) contains a single amino acid substitution abolishing residual transcriptional activation activity (Phe-442 to Pro-442) (Cress and Triezenberg 1991; Regier et al. 1993).

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

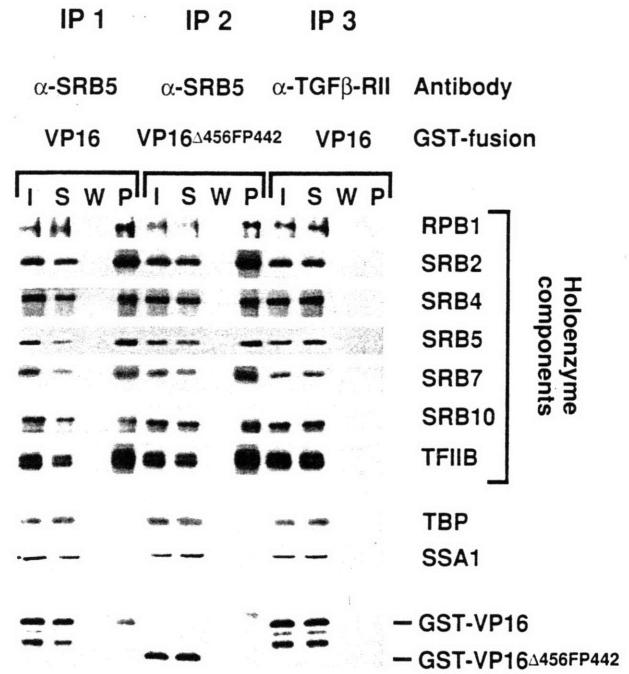

Figure 6. Coimmunoprecipitation of the VP16 activator with the RNA polymerase II holoenzyme. IP 1, IP 2, and IP 3 represent three separate immunoprecipitation experiments. A yeast whole cell extract was subjected to an ammonium sulfate cut and fractionated by step elution from Bio-Rex 70 and DEAE–Sephacel (Materials and methods); immunoprecipitations were carried out with anti-SRB5 antibodies (IP 1 and IP 2), or a control antibody, anti-hTGFβ-RII (IP 3). Recombinant GST–VP16 was added to the input of IP 1 and IP 3, whereas recombinant GST–VP16$^{\Delta 456FP442}$ was added to the input of IP 2. 1/50 of the input (I) and supernatant (S), and 1/5 of the last wash (W) and pellet (P) were subjected to SDS-PAGE and analyzed by Western blot using specific antibodies.

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

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

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

A VP16 column retains the mediator of activation

We investigated whether the mediator subcomplex interacts with VP16 and, if so, whether the interaction involves both VP16 activation subdomains. A mediator preparation was passed over columns containing GST–VP16, GST–VP16$^{\Delta 456}$, or GST–VP16$^{\Delta 456FP442}$. The columns were washed extensively and GST–fusion and associated proteins were eluted with glutathione. The onput, flowthrough, wash, and eluate fractions from the three columns were subjected to Western blot analysis (Fig. 8A). Components of the mediator were bound most effectively by GST–VP16. In contrast, there was limited binding of the mediator to GST–VP16$^{\Delta 456}$ and no apparent binding to GST–VP16$^{\Delta 456FP442}$. Figure 8B shows that the mediator preparation lacks RPB1, confirming that it contains little or no contaminating holoenzyme. These results indicate that the VP16 activator binds to the mediator. Furthermore, both of the activation subdomains of VP16 contribute to maximal binding of the mediator.

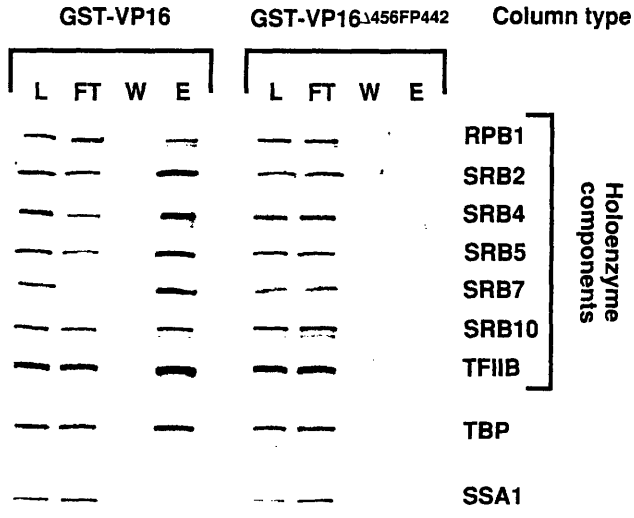

Figure 7. RNA polymerase II holoenzyme and TBP bind a VP16 activator column. The DEAE–Sephacel fraction described in Fig. 6 was loaded onto GST–VP16 and GST–VP16$^{\Delta 456FP442}$ glutathione–agarose columns. After washing, the columns were eluted with glutathione. 1/200 of the load (L) and the flowthrough (FT), and 1/10 of the last wash (W) and the eluate (E) were subjected to SDS-PAGE and analyzed by Western blot.

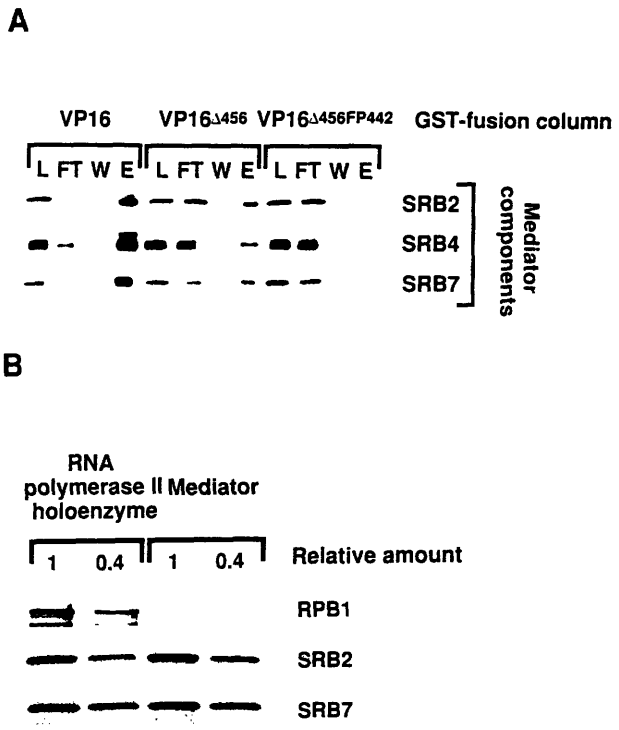

Figure 8. Mediator of activation binds to VP16 activator column. (*A*) Mediator of activation (peak fractions from heparin column) was loaded onto GST–VP16, GST–VP$^{\Delta 456}$, and GST–VP16$^{\Delta 456FP442}$ glutathione–agarose columns. After washing, the columns were eluted with glutathione. 1/150 of the load (L) and the flowthrough (FT), and 1/60 of the last wash (W) and the eluate (E) were subjected to SDS-PAGE and analyzed by Western blot. (*B*) Mediator of activation is depleted for RNA polymerase II. Mediator of activation (peak fractions from heparin column) and pure RNA polymerase II holoenzyme (Koleske and Young 1994) were analyzed by Western blot for the presence of the largest subunit of RNA polymerase II (RPB1) and for SRB2 and SRB7 proteins. The lanes contained 0.8 and 0.32 μg of RNA polymerase II holoenzyme, and 2.5 and 1 μg of mediator of activation, respectively.

Discussion

The genetic and biochemical analyses reported here provide a more complete description of the RNA polymerase II holoenzyme. The products of all nine *SRB* genes described thus far are components of the holoenzyme.

All nine SRB proteins can be dissociated from the holoenzyme in a subcomplex that functions in basal and activated transcription and has been termed the mediator of activation. The ability of holoenzymes to respond to activators suggests a model in which the holoenzyme is recruited to the initiation complex through direct interactions with activator proteins. We find that the VP16 activator can bind to the holoenzyme. Because VP16 can bind the mediator, this subcomplex is among the components of the holoenzyme that interact with VP16.

SRB components and the RNA polymerase II holoenzyme

The nine *SRB* genes were identified through a selection for genes whose products are involved in RNA polymerase II transcription in vivo. Suppressing alleles of the *SRB* genes were isolated by their ability to suppress the cold-sensitive phenotype of yeast cells that contain RNA polymerase II molecules with truncated CTDs. The isolation of the *SRB2, SRB4, SRB5,* and *SRB6* genes and the biochemical analysis of their protein products originally led to the discovery of the RNA polymerase II holoenzyme (Koleske and Young 1994). Two additional *SRB* genes, *SRB10* and *SRB11,* were described recently; they encode kinase and cyclin-like polypeptides that are tightly associated with the holoenzyme and appear to have roles in CTD phosphorylation (Liao et al. 1995). Analysis of all 75 independent suppressor isolates obtained in the genetic selection has led to the identification and characterization of three additional *SRB* genes. We have summarized the data for the nine *SRB* genes and proteins in Table 1.

Biochemical studies have shown that all nine SRB proteins are integral components of the RNA polymerase II holoenzyme. The SRB proteins copurify with RNA polymerase II, TFIIB, TFIIF, and TFIIH in conventional chromatography (Fig. 4A; Koleske and Young 1994). All SRB proteins tested (SRB2, SRB4, SRB5, SRB6, SRB7, SRB10, and SRB11) and other components of the holoenzyme have been shown to coimmunoprecipitate with SRB5 (Fig. 6A; Koleske and Young 1994; Liao et al. 1995). Furthermore, when we purify for any of these SRB proteins, all of the SRB protein detected by Western analysis copurifies with the holoenzyme (Koleske and Young 1994; Liao et al. 1995; C.J. Hengartner and A.J. Koleske, unpubl.).

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

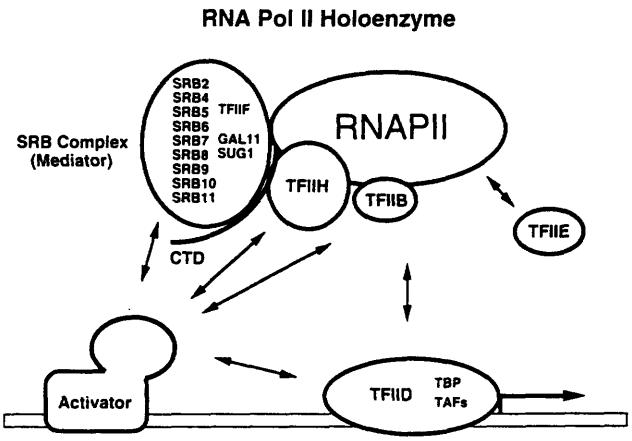

Figure 9. Model for interactions among activators, TFIID, and the RNA polymerase II holoenzyme.

Several lines of evidence suggest that the RNA polymerase II holoenzyme is the form of the enzyme involved in transcription initiation in vivo. Genetic analysis has indicated that the CTD and SRB gene products have roles in transcription initiation and are essential for normal yeast cell growth. Using temperature-sensitive mutants, we have found that SRB4 and SRB6 are necessary for transcription of most, if not all, genes transcribed by RNA polymerase II in vivo (Thompson and Young 1995). Because the vast majority of SRB protein in cell extracts is tightly associated with the RNA polymerase II holoenzyme, these results suggest that the holoenzyme is employed at most polymerase II promoters in vivo.

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

Initiation complex formation with the holoenzyme

The identification of a yeast RNA polymerase II holoenzyme suggests a model for initiation complex formation

in which the holoenzyme is the form of RNA polymerase recruited to promoters. Activators are believed to influence the rate of transcription of an adjacent gene in part by interacting with components of the initiation apparatus and affecting stable initiation complex formation. Figure 9 depicts a model that posits interactions between activators and TFIID and between activators and components of the holoenzyme. These interactions could occur sequentially or simultaneously.

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

The portion of the model in Figure 9 that depicts interactions between activators and the RNA polymerase II holoenzyme is supported by the following lines of evidence. The RNA polymerase II holoenzyme contains components necessary and sufficient for responding to transcriptional activators in a reconstituted system, apparently in the absence of TAFs (Kim et al. 1994; Koleske and Young 1994). Genetic evidence suggests that GAL11 and SUG1, two components of the mediator, may interact with certain activators (Nishizawa et al. 1990; Swaffield et al. 1992). We have shown that VP16 can bind to the mediator subcomplex of the holoenzyme. Thus, the activator VP16 has been shown to interact in vitro with three components of the RNA polymerase II holoenzyme: the mediator subcomplex, TFIIB (Lin et al. 1991; Goodrich et al. 1993) and TFIIH (Xiao et al. 1994).

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

Material and methods

Genetic manipulations

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

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

Yeast strains of the opposite mating type of approximately half of the dominant suppressors and half of the recessive suppressors were generated by inducing a mating type switch by expression of the *HO* gene placed on a plasmid under the control of a galactose-inducible promoter. Random spore analysis of the dominantly suppressing mutations was used to determine whether 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 was scraped off and shaken overnight at 30°C in 0.5 ml of 30 mM β-mercaptoethanol and 100 ng/ml of Zymolase 100 T (ICN). Added were 0.5 ml of 1.5% NP-40 and 0.4 gram of glass beads, and the mixture was incubated on ice for 15 min. The suspension was then vortexed for 3 min, incubated on ice for 5 min, vortexed for 2 min, and the glass beads were allowed to settle for 10 min at room temperature. The supernatant was removed and spun for 2 min, and the pellet was washed once in water, and resuspended in water and a portion plated onto YEPD. Approximately 50 of the haploid offsprings were assayed for their ability to grow at 12°C. If all haploids were able to grow at 12°C, then the two *SRB* isolates were assumed to contain mutations

Table 2. *Yeast strains*

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

Table 3. *Plasmids*

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

in the same gene. Genetic complementation of the recessive alleles involved mating haploids to each other, each containing the CTD truncation mutation *rpb1Δ*104 and an independently isolated *srb* mutation, to form diploids and assess 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 *rpb1Δ*104 and a recessive *srb* allele were unable to grow at 12°C and on pyruvate media when transformed with the corresponding wild-type *SRB* allele.

Deletions of *SRB7*, *SRB8*, and *SRB9* were created by a single-step disruption method (Rothstein 1991). Z558 was transformed with the desired DNA fragment and plated on SC − Ura media (Thompson et al. 1993). Southern analysis was used to confirm that a single copy of the desired *SRB* gene had been deleted. The diploid was sporulated and tetrads dissected (>20) on YEPD plates and scored for nutritional auxotrophies and growth at a variety of temperatures. Z697 was created by transformation with the *srb7Δ1*::URA3hisG fragment from pCH46. Two spores from each tetrad were viable and these spores were uracil auxotrophs, indicating that *SRB7* is essential. Z698 was created by transformation with the *srb8Δ1*::URA3hisG fragment from pSL315 and Z699 was created by transformation with the *srb9Δ1*::URA3hisG fragment from pCH66. In each case, segregants scored 2:2 for uracil prototrophy and all uracil prototrophs exhibited mild cold-sensitive, temperature-sensitive, and slow-growth phenotypes, indicating that *SRB8* and *SRB9* deletion strains are conditionally viable. *srb8Δ1* and *srb9Δ1* strains are also flocculent as are the suppressing isolates of *SRB8* and *SRB9*. Strains containing unmarked deletions of *SRB8* and *SRB9* were created by selecting for the excision of the *URA3* gene by growth on 5-FOA (Alani et al. 1987).

DNA methods

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

Cloning and sequence analysis

Genomic clones of *SRB7* (pCH2), *SRB8* (pSL301), and *SRB9* (pCH47) were isolated as described previously (Liao et al. 1995) by transformation and complementation of Z694, Z695, and Z696, respectively. When necessary, the wild-type genes were further localized by subcloning fragments of the genomic inserts and repeating the screen. The clones with the smallest inserts were sequenced. pCH36 was created from pCH7 in vivo by transforming Z694 with linearized pCH7 lacking *SRB7*-coding DNA and isolating the plasmid from a Ura$^-$ transformant that had repaired the plasmid with the mutant *srb7-1* sequences from the chromosome (Rothstein 1991). *SRB7* and *SRB9* were completely sequenced on each strand using genomic DNA from pCH7 and pCH47, respectively. Unidirectional deletions were constructed using the Erase-a-Base system (Promega), and double-stranded sequencing with dideoxynucleotides and Sequenase (U.S. Biochemical) was carried out as described by the manufacturer using T3 and T7 promoter primers. Gaps in the sequence were filled in by sequencing with internal oligonucleotide primers. Sequence comparison analysis was performed at the National Center for Biotechnology Information using the BLAST network service (Altschul et al. 1990).

The *srb7-1* mutant allele obtained by plasmid gap repair in vivo contained a single-point missense mutation changing Ala-21 to Thr (Fig. 1B). Unlike their wild-type counterparts, plasmids containing this mutation did not prevent growth at 12°C when transformed into yeast cells containing the CTD truncation mutation *rpb1Δ*104 and *srb7-1*(Z694), indicating that the correct gene was cloned. Similar results were obtained for the *srb8-1* and *srb9-1* mutant alleles obtained by plasmid gap repair in vivo.

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

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

Purification of recombinant proteins

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

GST–VP16, GST–VP16$^{\Delta 456}$, and GST–VP16$^{\Delta 456FP442}$ recombinant proteins were purified from DH5α carrying pGVP, pGVPΔ456, and pGVPΔ456–FP442 (provided by Michael Green,

University of Massachusetts, Worcester), respectively, as described (Smith and Johnson 1988).

Mediator purification and assay

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

Western blot analysis

Western blotting was performed by standard methods (Harlow and Lane 1988). RPB1 was detected via the CTD with 8WG16 monoclonal antibody ascites fluid (Promega). Polyclonal rabbit anti-SRB2, anti-GST-SRB4, anti-SRB5, anti-GST-SRB6, anti-SRB7, anti-SRB8 (amino acids 868 to 1226), anti-GST-SRB9 (amino acids 45 to 501), and anti-SRB10 (amino acids 1–271) antiserum were used to detect the SRBs. TBP, TFIIB, SSA1, and GST were detected using specific rabbit polyclonal antiserum. In all cases, bands were visualized by secondary probing with alkaline phosphatase conjugated secondary antibodies (Promega).

DEAE-400 fraction preparation

Two kilograms of dry yeast (Red Star) were disrupted essentially as described (Thompson et al. 1993), except that the disruption buffer was 1.2 M ammonium sulfate, 160 mM HEPES–KOH (pH 7.6), 4 mM DTT, 40 mM EDTA, 2 mM PMSF, 4 mM benzamidine, 2 μM pepstatin A, 0.6 μM leupeptin, and 2 μg/ml of chymostatin. After the initial centrifugation, 1/100 volume of 10% polymin P (pH 7.0) was added to the supernatant. The supernatant was incubated for 10 min at 4°C and spun for 20 min at 5000 rpm in a Sorvall H6000A rotor. Solid ammonium sulfate was added to 35% saturation, and the solution was incubated for 30 min. The suspension was centrifuged for 1 hr at 13,000 rpm in a GSA rotor. The pellet was resuspended in 9.5 liters of buffer A (20 mM HEPES–KOH at pH 7.6, 1 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 0.5 μM pepstatin A, 0.15 μM leupeptin, and 1 μg/ml of chymostatin) to a conductivity equal to buffer A–150 mM potassium acetate. The resuspended pellet was centrifuged for 20 min at 5000 rpm in a H6000A rotor. The supernatant (6.14 grams) was incubated for 1.5 hr with 500 grams of damp Bio-Rex 70 pre-equilibrated in buffer A–150 mM potassium acetate. The resin was collected by filtration, washed with 3 liters of buffer A–150 mM potassium acetate, and packed into a column (5 cm width). The column was eluted with buffer A–600 mM potassium acetate at a flow rate of 8 ml/min. The eluate (0.6 grams, 165 ml) was diluted with 500 ml of buffer B (20 mM Tris-acetate at pH 7.8, 1 mM EDTA, 20% glycerol, 0.01% NP-40, 1 mM DTT, and the same protease inhibitors as in buffer A). The diluted eluate was centrifuged for 10 min at 10,000 rpm in a GSA rotor. The supernatant was loaded onto a column (5 cm width) containing 100 ml of DEAE–Sephacel pre-equilibrated with buffer B–200 mM potassium acetate at a flow rate of 8 ml/min. The column was washed with 200 ml of buffer B–200 mM potassium acetate and eluted with buffer B–400 mM potassium acetate. The eluate contained 150 mg of protein in 60 ml.

Immunoprecipitation

The DEAE-400 fraction (0.1 ml) was dialyzed against modified transcription buffer (MTB) (50 mM HEPES–KOH at pH 7.3, 100 mM potassium acetate, 25 mM MgAc, 5 mM EGTA. 1 μM DTT, 10% glycerol, 0.01% NP-40, 1 mM PMSF, 2 mM benzamidine, 2 μM pepstatin A, 0.6 μM leupeptin, and 2 μg/ml of chymostatin) and subsequently diluted to 0.4 ml with MTB. One microgram of either affinity-purified anti-SRB5 antibodies or purified anti-human TGF-β–RII peptide (amino acids 30–44) IgG, and 1μg of recombinant GST–VP16 or GST–VP16$^{\Delta 456FP442}$ were added to the input. Immunoprecipitation was carried out essentially as described (Harlow and Lane 1988). Thirty microliters of anti-rabbit antibody linked to dynabeads (Dynal) was used as the secondary reagent in the immunoprecipitation.

Affinity chromatography

One milliliter of the DEAE-400 fraction (for holoenzyme binding experiments) or 1 ml of the mediator preparation (peak fractions from the heparin column) was dialyzed for 2 hr against MTB. The GST–VP16, GST–VP16$^{\Delta 456}$, and GST–VP16$^{\Delta 456FP442}$ affinity columns were prepared by immobilizing GST fusion proteins on glutathione–agarose beads as described (Smith and Johnson 1988). The columns contained 200 μl matrix and contained ~300 μg of GST fusion proteins. The columns were equilibrated with 6 ml MTB (minus DTT), loaded three times over with 0.2 ml of dialyzed DEAE-400 fraction (for holoenzyme binding experiments) or 0.37 ml of the dialyzed mediator preparation (0.25 mg/ml), washed with 4 ml of MTB (minus DTT) followed by 2 ml of MTB (minus DTT and NP-40), and eluted with buffer containing 50 mM Tris-Cl (pH 7.5), 0.1 mM DTT, and 5 mM glutathione, eluting both GST–fusion proteins and interacting proteins.

Acknowledgments

We thank Arun Patel for assisting in the cloning of *SRB8*, Young Joon Kim and Roger Kornberg for purified TFIIH, Michael Sayre for purified TFIIF, Ellen Gadbois and Peter Murray for anti-SSA1 and anti-GST polyclonal antisera, Harvey Lodish for purified anti-human TGFβ-receptor type II antibody, and Ellen Gadbois and Peter Murray for helpful discussions and comments on the manuscript. D.M.C. is a predoctoral fellow of the Howard Hughes Medical Institute and C.J.H. is a predoctoral fellow of the FCAR. Supported by grants from the National Institutes of Health.

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Note added in proof

The sequence data for *SRB7* and *SRB9* have been deposited to the GenBank data library under accession nos. U23811 and U23812, respectively.

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For CTD phosphorylation assays, core or holo-RNA polymerase II (60 and 150 ng, respectively), TFIIH (30 ng), and 0.3 mCi of [γ-^{32}P]ATP are incubated for 30 min at room temperature in 15 μl of 20 m*M* HEPES-KOH (pH 7.8), 7.5 m*M* magnesium acetate, 1 m*M* DTT, 1 m*M* ATP, and 120 m*M* potassium acetate, followed by addition of 5 μl of 5× SDS gel-loading buffer and analysis by SDS–PAGE. Incorporation of ^{32}P into the largest polymerase subunit, Rpb1, is quantified with the use of a PhosphorImager (Molecular Dynamics), calibrated by liquid scintillation counting.

[16] Purification of Yeast RNA Polymerase II Holoenzymes

By ANTHONY J. KOLESKE, DAVID M. CHAO, and RICHARD A. YOUNG

RNA polymerase II can be purified from yeast in a high molecular weight complex called an RNA polymerase II holoenzyme.[1,2] This form of RNA polymerase II appears to be responsible for transcription initiation *in vivo*.[3] The holoenzyme form of RNA polymerase II contains multiple general transcription factors and a large multisubunit regulatory subcomplex. This regulatory subcomplex, which can be dissociated from RNA polymerase II with antibodies directed against the enzyme, contains the general transcription factor TFIIF, the SRB (suppressor of RNA polymerase B) proteins, and additional regulatory proteins, and has been called the Mediator.[4] Transcription by the RNA polymerase II holoenzyme can be stimulated by activator proteins,[1] a property that can be reconstituted with purified RNA polymerase II, general transcription factors, and the Mediator subcomplex.[4]

There are three forms of yeast RNA polymerase II holoenzyme that have been detected thus far, each of which was purified on the basis of its association with SRB proteins. The largest form contains RNA polymerase II, the Mediator subcomplex, and the general transcription factors TFIIH and TFIIB.[1] A second form of holoenzyme can be purified that has all these components except TFIIB (D. Chao, unpublished observation, 1995) and the third form lacks TFIIB and TFIIH.[4] It seems likely that the isolation of the smaller forms of the holoenzyme is a consequence of holoenzyme

[1] A. J. Koleske and R. A. Young, *Nature (London)* **368**, 466 (1994).
[2] A. J. Koleske and R. A. Young, *Trends Biochem.* **20**, 113 (1995).
[3] C. M. Thompson and R. A. Young, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4587 (1995).
[4] Y. J. Kim, *et al.*, *Cell* 77(4), 599 (1994).

instability. Such instability may be due to strain background, growth conditions, and extract and purification procedures.

In this chapter, we describe methods for the purification and assay of the two largest forms of RNA polymerase II holoenzyme from the yeast *Saccharomyces cerevisiae*. Use of the purified holoenzyme for transcription studies *in vitro* should yield new and exciting clues regarding the mechanisms underlying transcriptional regulation.

Growth of Yeast

The distinguishing feature of an RNA polymerase II holoenzyme is its association with multiple SRB regulatory proteins. The SRB proteins are somewhat more abundant in yeast cells grown in minimal medium than they are in cells grown in rich medium and it is possible that the largest form of the holoenzyme is more stable in minimal medium.[2-5] The total yield of yeast grown in minimal medium is only about 4 g (wet weight)/liter, so considerable effort is necessary to prepare the 500 g of yeast that is typically employed in the purification described here. We have used three approaches to obtaining 500 g of cells: (1) prepare cells using the protocol described below; (2) grow cells in a fermentor in rich medium: and (3) purchase commercial yeast. Although it is considerably more laborious, we find that the first approach is the most dependable in generating high-quality yeast extracts.

To generate approximately 500 g of cells, we grow six 3-liter cultures at a time. About 70 g of yeast (wet pellet weight) is obtained from six 3-liter cultures, so this procedure is repeated until 500 g is obtained. Those unfamiliar with the growth of yeast may wish to consult a reference before getting started.[6] The protease-deficient yeast strain BJ926 can be used to minimize the protease content of whole-cell extracts.[7]

1. Two days before starting cultures, patch several single colonies of freshly streaked BJ926 onto a rich plate (YPD) using a sterile toothpick. Make dime-sized patches and incubate them at 30° overnight.

2. Inoculate each of two 300-ml cultures of YNB medium [0.15% (w/v) Difco (Detroit, MI) yeast nitrogen base, 0.5% (w/v) ammonium sulfate, 200 m*M* inositol, 2% (w/v) glucose] in 1-liter flasks with the yeast scraped from a single patch with a sterile stick. Grow these cultures at 30°, shaking at approximately 200 rpm, for 20–24 hr.

[5] C. M. Thompson, A. J. Koleske, D. M. Chao, and R. A. Young, *Cell* **73**, 1367 (1993).
[6] F. Sherman, *Methods Enzymol.* **194**(1), (1991).
[7] E. W. Jones, *Methods Enzymol.* **194**(1), 428 (1991).

3. Inoculate each of six 3-liter cultures of 1× YNB (in 6-liter flasks) with 100 ml of the 24-hr culture. Allow the yeast to grow at 30°, shaking at 200 rpm, to an OD_{600} of 4.0 to 4.5 (20–24 hr).

4. Yeast cells are collected by centrifugation in six 1-liter bottles at 4000 rpm in a Sorvall (Newtown, CT) RC3B or similar centrifuge for 10 min at 4°. The supernatant can be carefully removed from the pelleted cells and another liter of culture can be added to the bottle and spun down on top of the previous yeast pellet. The pellets should be washed by resuspending in 100–200 ml of ice-cold buffer [20 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–KOH (pH 7.5), 10% (v/v) glycerol, 50 m*M* potassium acetate, 1 m*M* dithiothreitol (DTT), and 1 m*M* EDTA]. This yeast suspension can be combined into one or two bottles and collected by centrifugation as described above for storage at −70°. Following centrifugation, discard the supernatant, weigh the bottle containing the pellet, and store it at −70° until enough yeast has been collected to proceed to the extraction process.

Yeast Extract Preparation

The following two procedures for producing yeast extracts can be adapted to 0.5–5 kg of yeast or even more. Owing to the labor involved in the purification, we do not recommend beginning with less than 500 g of yeast (wet pellet weight).

Procedure 1

In this procedure, the yeast whole-cell extracts are generated using methods similar to those described by Sayre *et al.*[8] The following protease inhibitors are used where indicated: 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 2 m*M* benzamidine, 2 μ*M* pepstatin A, 0.6 μ*M* leupeptin, chymostatin (2 μg/ml), antipain hydrochloride (5 μg/ml; Sigma, St. Louis, MO). All procedures are carried out at 4°, unless otherwise noted.

1. Remove the yeast pellet from storage to 4° for about 1 hr to allow the pellet to thaw partially.

2. Resuspend the pellet in 3× lysis buffer plus protease inhibitors, using 0.5 ml/g (wet weight) of yeast. The pellet can be resuspended using a rubber policeman or by vigorous swirling of the bottle.

3. Rinse acid-washed glass beads (400–600 μm; Sigma) with 1× lysis buffer [20% (v/v) glycerol, 50 m*M* potassium acetate, 150 m*M* Tris–acetate (pH 7.9), 1 m*M* EDTA, 2 m*M* DTT].

[8] M. H. Sayre, H. Tschochner, and R. D. Kornberg, *J. Biol. Chem.* **267**(32), 23376 (1992).

4. Add glass beads and the cell suspension to a Bead-beater (Biospec Products). Add the glass beads to the chamber of the Bead-beater until it is 50–60% full. Fill the remaining space with the yeast–lysis buffer suspension. Assemble the chamber and seal. Fill the reservoir with an ice–salt–water bath. Use about 40 g of NaCl for the bath.

5. Run the beater 20 times for 30 sec. Allow 90 sec between runs to permit cooling of the chamber. Add more ice during the run if necessary. A digital controller/timer (VWR Scientific) is useful for automating this step.

6. Disassemble the chamber. Let the beads settle to the bottom and remove the lysate while minimizing the amount of bead carryover. Although tedious, pipetting the lysate works well. Rinse the beads further with 30–60 ml of 3× lysis buffer and decant the supernatant. The chamber can be refilled with yeast–lysis buffer solution for another round of lysis. The beads should be discarded after two rounds of lysis.

7. Spin the lysate in a Sorvall GSA rotor at 10,000 rpm for 20 min at 4° to pellet cell debris.

8. Adjust the supernatant slowly to 0.5 *M* in potassium acetate using a stock solution of 5 *M* potassium acetate with stirring, and stir gently for 30 min.

9. Spin the extract at 41.000 rpm in a Beckman (Fullerton, CA) Ti 50.2 rotor for 90 min. The lipid at the top of the tube is removed with a cotton-tipped applicator or by aspiration. Carefully remove the supernatant by pipetting, taking care to avoid the opaque and brown material at the bottom of the tube. The yield should be about 340–370 ml of 20- to 25-mg/ml protein extract for 500 g of starting material. Extracts can be frozen at −70°.

Procedure 2

The freeze-thaw procedure described below is extensively modified from the procedure of Dunn and Wobbe.[9] This procedure requires large amounts of liquid nitrogen, and it is important to take appropriate safety precautions. Handle the liquid nitrogen in a well-ventilated area, use appropriate containers, and wear protective clothing, glvoes, and safety goggles. Because this procedure has the potential to be quite messy, generous use of bench paper is recommended. Note that the form of holoenzyme purified by this procedure from BJ926 cells grown in rich medium does not contain TFIIB. All steps in this procedure are at room temperature unless otherwise specified.

[9] B. Dunn and C. R. Wobbe, Cell disruption using liquid nitrogen. *In*: "Current Protocols in Molecular Biology" (F. M. Ausubel *et al.*, eds.), pp. 13.5–13.6. John Wiley & Sons, New York, 1994.

1. Mix 500 g of frozen yeast with 500 ml of buffer containing 100 m*M* potassium acetate, 40 m*M* K-HEPES (pH 7.6), 2 m*M* Na-EDTA, 2 m*M* DTT plus 2× protease inhibitors, and thaw the mixture in a 65° water bath. Do not allow the temperature of the suspension to exceed 4°.

2. Pour the thawed suspension into 4-liter containers of liquid nitrogen while stirring with a plastic pipette. Pour the suspension into the liquid nitrogen at a speed that yields popcorn-size pellets of frozen cells. Drain the excess liquid nitrogen from the frozen pellets.

3. Place the frozen pellets in the 1-liter stainless steel chamber of a commercial Waring blender (VWR Scientific) until it is half full. Replace the lid, and blend for 2–4 min until the mixture has the consistency of a fine powder. It may be necessary to stir the mixture occasionally with a pipette to prevent clumping. Repeat the blending until all of the cells have been processed.

4. Thaw the disrupted cells at 65° in a beaker with stirring until the extract has the consistency of a viscous milkshake. In 750-ml batches, blend the lysate for 10 sec; wait 10 sec, and blend again for 10 sec.

5. Repeat steps 2–4. The number of cycles required for maximal cell breakage depends on the strain and growth conditions. For example, commercial yeast (Red Star) rehydrated for 30 min in 2% (w/v) glucose requires only one cycle, while BJ926 grown in a fermenter requires four cycles.

6. Spin the extract in a Sorvall H6000A rotor at 5000 rpm for 1 hr at 4°. Decant the supernatant and pour it through cheesecloth. Add 1/8 vol of glycerol and 1/8 vol of 5 *M* potassium acetate and stir at 4° for 15 min.

7. Spin the extract for 30 min at 13,000 rpm in a Sorvall GSA rotor at 4°. Pour the supernatant through cheesecloth at 4°. At this point, the extract can be stored temporarily at −70° if necessary.

8. Spin the extract for 90 min at 4° at 40,000 rpm in a Beckman 50 Ti rotor or at 42,000 rpm in a Beckman 45 Ti rotor. Remove lipids by aspiration, and recover the supernatant by pipetting.

9. The typical yield for this extraction procedure is 400 ml, with a protein concentration of 25–35 mg/ml. Extracts can be frozen at −70°.

Chromatography

The steps outlined here are scaled for 400 ml of extract with a protein concentration of approximately 25 mg/ml. The fractions can be stored at −20 or −70° between any chromatographic step in this sequence.

1. Prepare a column with Bio-Rex 70 resin 100–200 mesh (Bio-Rad, Richmond, CA). It is important to prepare this resin according to manufacturer directions and to equilibrate it with buffer A (100). Buffer A contains

20% (v/v) glycerol, 20 m*M* HEPES–KOH (pH 7.5), 1 m*M* DTT, 1 m*M* EDTA, and protease inhibitors. Buffer A (100) is buffer A plus 100 m*M* potassium acetate. As the protein is eluted in steps from this column, the exact column dimensions are not important; however, wider columns (>4-cm diameter) facilitate a faster flow rate. We use a 5 × 17 cm column. Typically, the column is loaded at 20–25 mg of protein per milliliter of column bed. Total onput onto this column should not exceed 50 mg of extract protein per milliliter of column bed.

2. If using an extract obtained with procedure 1, dilute the whole-cell extract 1:5 in buffer A so that the potassium acetate concentration is reduced to 100 m*M*. Load the extract onto the Bio-Rex 70 (Bio-Rad) column at a flow rate of 10 ml/min. Wash the column with buffer A (100) until no further protein can be eluted from the column, usually two to three column volumes. The protein concentration in the column eluate should be monitored by mini-Bradford assays, using a minimum of each fraction. The Bio-Rad protein assay is convenient for this; 10 μl of each fraction is added to 325 μl of Bio-Rad protein assay solution.

3. Elute the column with a step wash of buffer A (300) (buffer A plus 300 m*M* potassium acetate) until no further protein elutes from the column (approximately two to three column volumes). Although the Bio-Rex 70 flowthrough and buffer A (300) fractions are not used further in this protocol, they may provide a source of other factors and can be saved at −70°.

4. Elute the column with a step wash of buffer A (600) until no further protein elutes from the column. The RNA polymerase II holoenzyme should elute in this step. The holoenzyme is not yet sufficiently purified to be assayed for activity at this stage; it can be assayed for activity after chromatography on hydroxylapatite (see step 8). The presence of the holoenzyme can be monitored by Western blot using antibodies to SRB2, SRB4, SRB5, and SRB6. RNA polymerase II can be assayed in a similar fashion using a monoclonal antibody directed against the largest subunit of RNA polymerase II (Promega, Madison, WI). The column can be further eluted with a step wash of buffer A (1000). Eighty percent of the holoenzyme eluting from the column should be in the buffer A (600) fraction.

5. Dilute the Bio-Rex 70 (600) fraction 1:6 with buffer B [20% (v/v) glycerol, 20 m*M* Tris–acetate (pH 7.9), 1 m*M* DTT, 1 m*M* EDTA, 0.01% (v/v) Nonidet P-40 (NP-40) and protease inhibitors] to reduce the salt concentration to 100 m*M*. Load the solution onto DEAE-Sephacel (Pharmacia, Piscataway, NJ) resin at a flow rate of 4 ml/min. Again, column shape is not particularly important here, as stepwise elutions are used; we use a 2.5 × 8.5 cm column. The column should contain about 1 ml of bed volume per 5 mg of onput protein.

6. Wash the column extensively with buffer B (100) (buffer B plus 100 m*M* potassium acetate) and then elute with step washes of buffer B (400) and buffer B (650). The holoenzyme will elute from this column in the 400 m*M* potassium acetate step and can be monitored by Western blot analysis. TFIIE, which is not a component of yeast holoenzymes and is required for assays of holoenzyme activity, can be prepared from the 650 m*M* eluate.[10]

7. The next step involves chromatography on a BioGel HTP hydroxylapatite column. Prepare the resin by incubating in buffer C [20% (v/v) glycerol, 10 m*M* potassium phosphate (pH 7.7), 100 m*M* potassium acetate, 1 m*M* DTT, 0.01% (v/v) NP-40, and protease inhibitors]. It will take approximately 10 ml to swell 1 g of BioGel HTP.

8. Load the DEAE-Sephacel buffer B (400) fraction directly onto a 1.5 × 6.5 cm BioGel HTP hydroxylapatite column at a flow rate of 1 ml/min. The fraction does not need to be dialyzed or diluted prior to loading onto the column. Wash the column with 20 ml of buffer C (EDTA). Buffer C is 20% (v/v) glycerol, 10 m*M* potassium phosphate (pH 7.7), 100 m*M* potassium acetate, 1 m*M* DTT, 0.01% (v/v) NP-40, and protease inhibitors. Buffer C (EDTA) contains 0.25 m*M* EDTA. Elute with a 120-ml linear gradient of buffer C (EDTA) to buffer D [buffer D is identical to buffer C (EDTA) except that it contains 300 m*M* potassium phosphate, pH 7.7]. Assay fractions for transcriptional activity (see below) and for the presence of holoenzyme protein, using Western blot analysis. The holoenzyme should elute from this column in a peak corresponding to 68 to 112 m*M* potassium phosphate.

9. Dialyze the holoenzyme peak from the BioGel HTP (Bio-Rad) against buffer E (100). Buffer E is the same as buffer B except the EDTA is 0.25 m*M*; buffer E (100) is buffer E plus 100 m*M* potassium acetate. Centrifuge the dialyzed material in a Sorvall SS34 rotor at 10,000 rpm for 20 min at 4°. Load the supernatant (approximately 11 mg protein in 20 ml) onto a Mono Q HR 5/5 FPLC column (Pharmacia) at a flow rate of 0.5 ml/min. Elute the column with a 15-ml linear gradient from buffer E (100) to buffer E (2000). Assay fractions for transcriptional activity and the presence of holoenzyme protein. The holoenzyme elutes from this column at 0.95 *M* potassium acetate.

10. Pool the peak fractions containing holoenzyme activity and dilute them 1:6 with buffer F. Buffer F is the same as buffer A except the EDTA concentration is 0.25 m*M*. Load this material (approximately 1.1 mg of protein in 10 ml) onto a Mono S HR 5/5 FPLC column (Pharmacia), and elute with a 10-ml gradient from buffer F (100) to buffer F (1000) at a flow

[10] M. H. Sayre, H. Tschochner, and R. D. Kornberg, *J. Biol. Chem.* **267**(32), 23383 (1992).

rate of 0.5 ml/min. Assay fractions for transcriptional activity and the presence of holoenzyme protein. The holoenzyme should elute from this column at 450 m*M* potassium acetate.

11. Dilute the pooled peak fractions (approximately 0.6 mg in 8 ml) 1:4 in buffer E and immediately load onto a 1.5 × 1.5 cm DEAE-Sephacel column, wash with buffer E, and elute with a 12-ml gradient from buffer E (100) to buffer E (1000) at a flow rate of 0.3 ml/min. Assay fractions for transcriptional activity and the presence of holoenzyme protein. The holoenzyme elutes from this column at 400 m*M* potassium acetate.

This holoenzyme preparation should be approximately 90% pure. The total yield of the holoenzyme should be about 0.5 mg and the purification should be approximately 10,000-fold. Holoenzyme preparations have been stored in this buffer at −70° for up to 2 years without appreciable loss of activity.

Assay for Holoenzyme Activity

All forms of RNA polymerase II holoenzyme described thus far lack the general transcription factors TBP and TFIIE, and these factors must be added to obtain transcription *in vitro*. Protocols for the purification of recombinant TBP and yeast TFIIE have been published.[10,11] TFIIB is also required for transcription by some holoenzyme preparations. Holoenzyme and factor preparations should be dialyzed against a low-salt buffer, such as buffer F, to reduce the salt content prior to assay. Alternatively, the salt concentration can be determined by conductivity measurements, and the salt concentration in the transcription assay buffer can be adjusted accordingly. The following assay is a modification of that described in Ref. 12.

1. Make up 2× transcription buffer. 1× transcription buffer contains 50 m*M* HEPES-KOH (pH 7.3), 100 m*M* potassium glutamate, 15 m*M* magnesium acetate, 5 m*M* EGTA, 100 ng of GAL4 G-template, 3 m*M* DTT, enzyme-grade acetylated bovine serum albumin (BSA, 50 μg/ml; Promega), and 10% (v/v) glycerol. Transcription buffer is made up at 2× concentration to allow for the addition of further components (holoenzyme, TBP, TFIIE, activator, etc.). If TBP, TFIIE, or holoenzyme are in solutions containing glycerol, the glycerol content in the reaction must be adjusted accordingly.

2. Assemble the transcription reactions. The transcription reactions will have a 25-μl final volume, but for preincubation, they are assembled in a

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[12] N. F. Lue and R. D. Kornberg, *Proc. Natl. Acad. Sci. U.S.A.* **84**(24), 8839 (1987).

volume of 22 μl at 24° and contain 20–40 ng of TBP, 20–40 ng of TFIIE, and 1 μg of holoenzyme. The remaining 3 μl of volume will contain the nucleotides added to initiate the reactions following the preincubation. Activators such as GAL4-VP16 are added at a concentration of 10 μM before the preincubation step.

3. Preincubate the reactions at 24° for 1 hr.

4. Initiate the transcription reactions by addition of 3 μl of 3.33 mM ATP, 3.33 mM CTP, 15 μM UTP, and [α-^{32}P]UTP (1.65 mCi/ml; 3000 Ci/mmol). The reactions are incubated at 24° for 40 min.

5. Terminate the transcription reaction by adding 100 μl of 10 mM Tris 8.0, 5 mM EDTA, 0.3 M NaCl, and RNase T_1 (2000 units/ml; Mannheim, Indianapolis, IN).

6. Prepare the transcripts for analysis by extracting the reactions twice with a 1:1 mixture of Tris-buffered phenol and chloroform. Precipitate RNA by addition of 2.5 vol of ethanol and incubation on dry ice–ethanol for 20 min, and pellet by high-speed centrifugation in a microcentrifuge for 15 min. Wash the pellet with 70% (v/v) ethanol. Allow to dry for several minutes and resuspend it in 10 μl of sample buffer [0.1× TBE, 80% (v/v) formamide, 0.01% (v/v) xylene cyanol, and 0.01% (w/v) bromphenol blue].

7. Analyze the transcripts by subjecting them to electrophoresis on a 6% (w/v) acrylamide [19:1 (v/v) acrylamide:bisacrylamide ratio]–7 M urea gel in 1× TBE buffer at 30 V/cm. Heat the resuspended pellets at 80° for 10 min, cool them briefly on ice, and spin to collect any condensation prior to loading the samples. The gel should be prerun at least 15–30 min before loading samples. Following electrophoresis, dry the gel and expose to autoradiography. The transcripts from the GAL4 G-less template are 350–375 nucleotides long.

Acknowledgments

We thank Sha-Mei Liao, who initiated biochemical analysis of transcription in the Young laboratory. We also thank Steve Buratowski, John Feaver, Michael Sayre, and Alan Sachs for reagents and advice on chromatography and Phil Johnson and John Harper for growing the yeast used in some of these experiments. D.M.C. is a predoctoral fellow of the Howard Hughes Medical Institute.

A kinase–cyclin pair in the RNA polymerase II holoenzyme

Sha-Mei Liao*, Jianhua Zhang*, Douglas A. Jeffery*, Anthony J. Koleske*, Craig M. Thompson*, David M. Chao*, Marinda Viljoen*†, Hendrik J. J. van Vuuren† & Richard A. Young*

* Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, Massachusetts 02142, and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA
† Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa

THE RNA polymerase II holoenzyme consists of RNA polymerase II, a subset of general transcription factors, and regulatory proteins known as SRB proteins[1,2]. The genes encoding SRB proteins were isolated as suppressors of mutations in the RNA polymerase II carboxy-terminal domain (CTD)[3,4]. The CTD and SRB proteins have been implicated in the response to transcriptional regulators[1–11]. We report here the isolation of two new *SRB* genes, *SRB10* and *SRB11*, which encode kinase- and cyclin-like proteins, respectively. Genetic and biochemical evidence indicates that the SRB10 and SRB11 proteins form a kinase–cyclin pair in the holoenzyme. The SRB10/11 kinase is essential for a normal transcriptional response to galactose induction *in vivo*. Holoenzymes lacking SRB10/11 kinase function are strikingly deficient in CTD phosphorylation. Although defects in the kinase substantially affect transcription *in vivo*, purified holoenzymes lacking SRB10/11 kinase function do not show defects in defined *in vitro* transcription systems, suggesting that the factors necessary to elicit the regulatory role of the SRB10/11 kinase are missing in these systems. These results indicate that the SRB10/11 kinase is involved in CTD phosphorylation and suggest that this modification has a role in the response to transcriptional regulators *in vivo*.

To identify new components of the RNA polymerase II holoenzyme, we isolated extragenic suppressors of a *Saccharomyces cerevisiae* RNA polymerase II CTD truncation mutation[4]. Recessive suppressing mutations were identified in two genes, *SRB10* and *SRB11*. Genetic analysis indicated that the CTD and the two *SRB* gene products are involved in the same process in transcription initiation. The mutant alleles *srb10-1* and *srb11-1* suppressed the conditional phenotypes of CTD truncation mutations but not the conditional phenotypes of other RNA polymerase II mutations. Genomic DNA clones containing *SRB10* and *SRB11* were isolated by genetic complementation and the complementing clones with the smallest inserts were sequenced (Fig. 1).

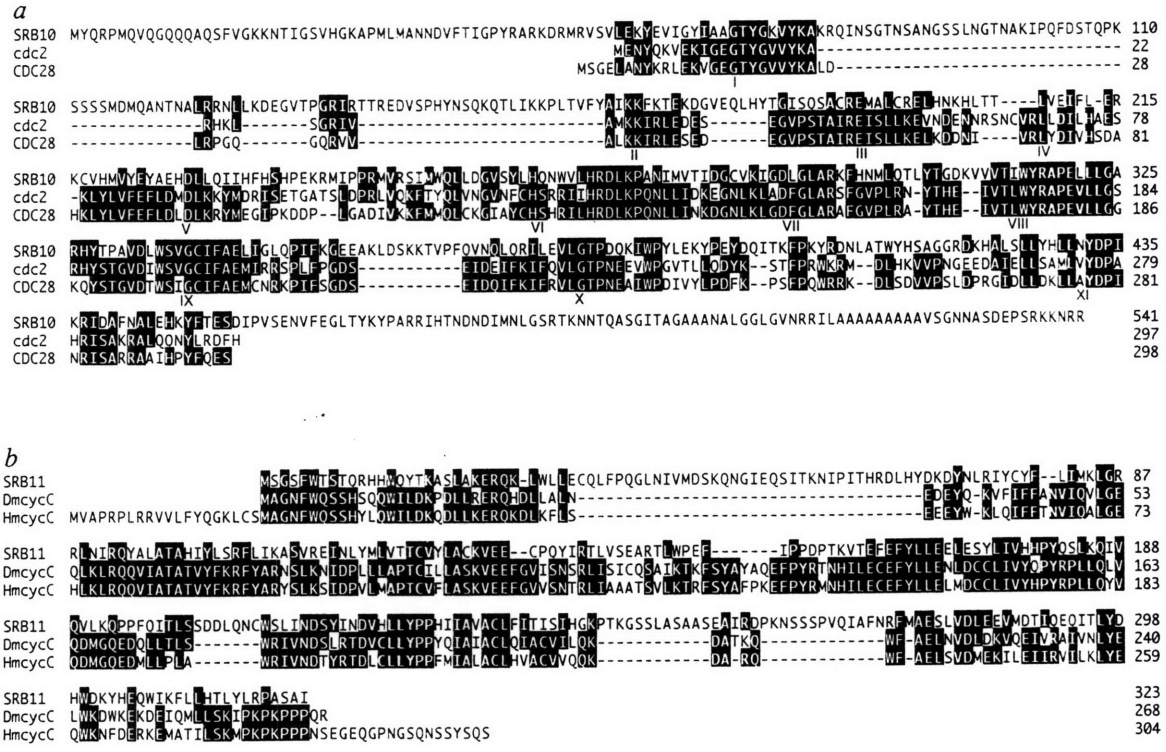

FIG. 1 Sequence of SRB10 and SRB11 proteins and alignment with related kinases and cyclins. *a*, SRB10 sequence alignment with the *S. pombe* Cdc2 and *S. cerevisiae* CDC28 proteins. Identical amino-acid residues are shown as white letters on a black background. Roman numerals indicate the 11 conserved kinase subdomains. Primer extension analysis of poly(A)$^+$ mRNA revealed that the *SRB10* transcript is initiated at position +12 in the open reading frame originally predicted for *UME5* (ref. 13). Thus the amino-acid sequence of SRB10/UME5 shown here lacks the 14 amino-terminal amino acids originally reported for UME5[13]. *b*, SRB11 sequence alignment with *Drosophila* (DmcycC) and human (HmcycC) cyclin C proteins.

METHODS. Genomic DNA clones containing *SRB10* and *SRB11* were isolated by exploiting their ability to reverse the suppressing phenotype of the recessive *srb* alleles in cells containing the truncated CTD allele (*rpb1Δ104*). A wild-type genomic DNA library constructed in a yeast *URA3* centromeric plasmid[4] was transformed into yeast cells containing the CTD truncation mutation *rpb1Δ104* and *srb10-1* or *srb11-1*. Ura$^+$ transformants were then screened for phenotypes characteristic of the *rpb1Δ104* CTD truncation mutant: lack of growth at 12 °C and inability to use pyruvate as a carbon source. Genomic clones containing *SRB10* (pSL201) and *SRB11* (pJZ11-1) were sequenced. *SRB10* and *SRB11* were physically mapped to chromosome XVI (λ clones 3168 and 4122) and to chromosome XIV (λ clone 3634), respectively, using the prime λ clone grid filters of the yeast genome (from L. Riles and M. Olson). Sequence comparison and alignments were made with the BLAST network service and the DNA Star Megalign program, respectively. The nucleotide sequence data for *SRB10* and *SRB11* can be found in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession numbers U20222 and U20221, respectively.

SRB10 encodes a 541-amino-acid protein kinase related to the Cdc2 kinases[12] (Fig. 1*a*). *SRB10* is identical to *UME5*, described recently as a regulator of meiosis-specific genes[13]. SRB10/UME5 protein sequence is ~40% identical to the *S. cerevisiae* CDC28 and *S. pombe* Cdc2 proteins and highly similar to PHO85, CTK1 and KIN28 (refs 14–16, respectively), members of the yeast Cdc2-related subfamily of serine/threonine protein kinases.

SRB11 encodes a 323-amino-acid protein which is ~28% identical and 58% similar in sequence to the human[17] and *Drosophila*[18] cyclin C proteins (Fig. 1*b*). Residues 75–184 of SRB11 are 37% identical to those of the 'cyclin box' of the human and *Drosophila* proteins; this region is conserved in cyclins and may be involved in the interaction between kinase and cyclin subunits[19–21].

We investigated whether SRB10 and SRB11 could be components of the RNA polymerase II holoenzyme. Western blot analysis with anti-SRB10 antibodies revealed that essentially all of the SRB10 in cell extracts copurified with the RNA polymerase II holoenzyme at each step of the purification procedure. The results in Fig. 2*a* show that RNA polymerase II and the SRB2, SRB4, SRB5, SRB6 and SRB10 proteins co-elute in the final purification step of the holoenzyme. Because we were unable to generate anti-SRB11 antibodies, an independent purification was carried out using a lysate from cells containing epitope-tagged SRB11; all of the epitope-tagged SRB11 copurified precisely with the RNA polymerase II holoenzyme (Fig. 2*a*). Thus, essentially all of the SRB10 and SRB11 in cell extracts is associated with the holoenzyme. Immunoprecipitation experiments confirmed that SRB10 and SRB11 are tightly associated with the holoenzyme (Fig. 2*b*). Thus, genetic and biochemical analysis indicates that all six SRB proteins identified so far (SRB2, 4, 5, 6, 10 and 11) are components of the transcription initiation complex, the RNA polymerase II holoenzyme.

The sequences of *SRB10* and *SRB11*, together with genetic evidence that the two gene products are involved in the same function, indicate that they may form a kinase–cyclin pair. The recombinant proteins bound to one another on a column (Fig. 2*c*) and interacted in a two-hybrid system[22] (data not shown), indicating that SRB10 and SRB11 are components of a kinase–cyclin pair in the holoenzyme.

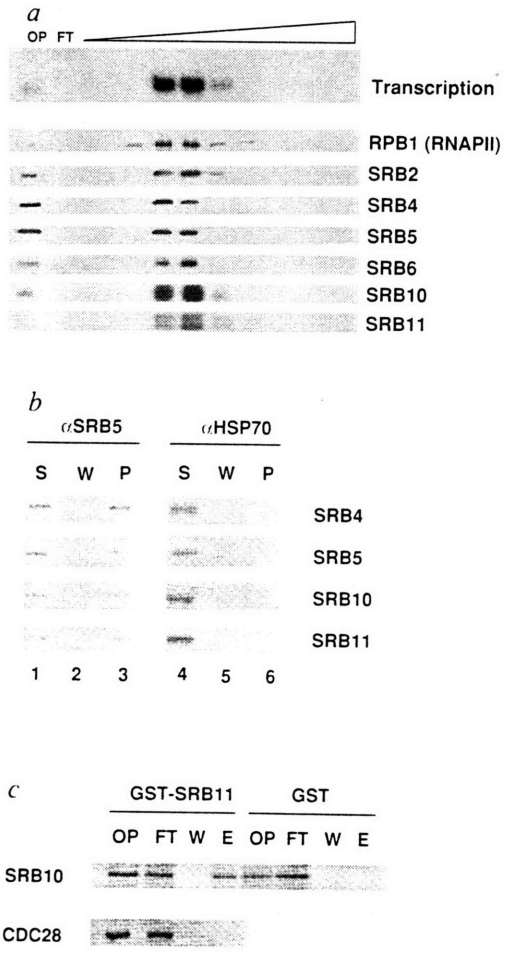

FIG. 2 SRB10 and SRB11 are components of the RNA polymerase II holoenzyme. *a*, RNA polymerase II holoenzyme was purified as described[1]. Holoenzyme loaded onto a Mono-S column, the last chromatographic step in the purification procedure, was eluted with a 0.1–1.0 M gradient of potassium acetate. The onput (OP) and flow through (FT) and a portion of every other fraction eluting between 0.1 and 0.9 M potassium acetate were analysed for holoenzyme activity (top panel) and for the presence of RNA polymerase II and SRB proteins by western blot analysis. The western blot for SRB11 was done with an RNA polymerase II holoenzyme purified independently from cells with an epitope-tagged SRB11 protein; the purification and transcriptional properties of this holoenzyme were identical to the holoenzyme lacking the epitope tag. *b*, Co-immunoprecipitation of SRB4, SRB10 and SRB11 with SRB5. Purified RNA polymerase II holoenzyme was immunoprecipitated using affinity-purified anti-SRB5 or anti-HSP70 antibodies. The supernatant (S), wash (W) and precipitate (P) were analysed by western blotting using antibodies against specific SRB proteins. Lanes: 1 and 4, supernatant from immunoprecipitation with anti-SRB5 and anti-HSP70 antibodies; 2 and 5, washes of immunoprecipitated material; 3 and 6, precipitated material. *c*, Recombinant SRB10 interacts with a glutathione-S-transferase (GST)–SRB11 fusion protein in affinity chromatography. Radiolabelled SRB10 or CDC28 was produced in an *in vitro* transcription/translation system and loaded onto GST–SRB11 and GST columns. Fractions were subjected to SDS–PAGE. OP, 0.5% of onput; FT, 0.5% of flowthrough; W, 10% of final wash; E, 10% of elution with reduced glutathione.

METHODS. Holoenzyme purification and *in vitro* transcription assays have been described[1]. The mAb 8WG16 (Promega) was used to detect RPB1 in western blots, rabbit anti-SRB antibodies to detect SRB2, SRB4, SRB5, SRB6 and SRB10, and mAb 12CA5 to detect an influenza haemagglutinin (HA) epitope tag introduced at the N terminus of SRB11[26]. Bands were visualized by secondary probing with alkaline-phosphatase-conjugated secondary antibodies (Pierce) or by chemiluminescence with horseradish-peroxidase-conjugated secondary antibody (Amersham). Immunoprecipitations were done with holoenzyme purified from an HA-tagged SRB11 strain (Z689) in buffer containing 50 mM HEPES-KOH, pH 7.3, 15 mM magnesium acetate, 100 mM potassium acetate, 1 mM EGTA, 10% glycerol, 0.1 mM DTT, 0.1% NP-40. Anti-rabbit antibody linked to dynabeads (Dynal) was used as the secondary reagent in the immunoprecipitation. For column chromatography, a GST–SRB11 fusion was constructed (pMV123) using pGEX-2T (Pharmacia) and recombinant GST–SRB11 and GST were purified from *E. coli* as described[27]. Columns containing 250 µl GST-beads were loaded with 40–50 µg GST–SRB11 or GST, and equilibrated with transcription buffer[8]. 25 µl labelled *in vitro* translated SRB10 or CDC28 (Promega TNT coupled system) was incubated with the immobilized proteins overnight at 4 °C, washed with transcription buffer +1% Triton X-100, and eluted with 10 mM reduced glutathione, 50 mM Tris-HCl, pH 8, 1% TritonX-100, 0.1 mM PMSF. Figures were prepared from digital replicas of primary data scanned using a UMAX UC840 Max Vision digital scanner.

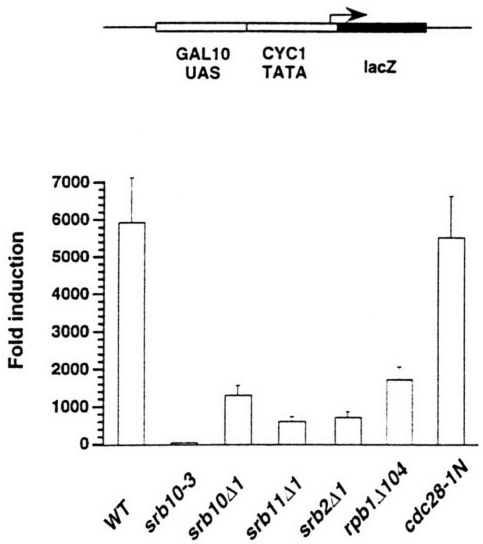

FIG. 3 *SRB10* and *SRB11* mutants are deficient in transcriptional responses at a GAL10 promoter *in vivo*. The relative ability of wild-type cells and *srb10* and *srb11* mutants to respond to galactose induction was measured by expressing *lacZ* under the control of a *GAL10* UAS-containing promoter, as described[6]. Extracts were prepared from induced or uninduced cells and the results of β-galactosidase assays are expressed as fold induction. Strains assayed were wild type (WT); Z690 (*srb10-3*) cells encoding an SRB10 protein in which Asp 290 had been replaced with Ala; Z687 (*srb10Δ1*) cells in which *SRB10* is deleted; Z688 (*srb11Δ1*) cells in which *SRB11* is deleted; Z437 (*srb2Δ1*) cells in which *SRB2* is deleted; Z551 (*rpb1Δ104*) cells encoding an RNA polymerase II CTD truncation mutant with 11 heptapeptide repeats; L5191 (*cdc28-1N*) cells encoding a mutant form of CDC28. METHODS. The *GAL10* UAS–*CYC1*–*lacZ* fusion plasmid pLGDS5, galactose induction procedures and β-galactosidase assay have been described[6]. Complete deletions of the *SRB10* and *SRB11* coding sequences (*srb10Δ1* and *srb11Δ1*) were constructed using a single-step disruption method[28]. The gene encoding the SRB10 D290A mutation (*srb10-3*) was constructed by oligonucleotide mutagenesis, and the Z690 mutant was generated by replacing the wild-type *SRB10* gene with *srb10-3*, as described[28]. The *srb10* and *srb11* mutant strains are conditionally viable; they exhibit mild cold-sensitive, temperature-sensitive, and slow growth phenotypes.

Mutations in the RNA polymerase II CTD and in SRB2 reduce the response of the transcription apparatus to regulators *in vivo*[3–7], so we investigated the effects of mutations in *SRB10* or *SRB11 in vivo* (Fig. 3). A β-galactosidase reporter vector with a *GAL10* UAS-containing promoter was introduced into cells with CTD and *srb* mutations and the cells were subjected to induction by galactose. The results revealed that cells lacking SRB10 or SRB11 function respond poorly to galactose. The defect was most pronounced (100-fold) in cells containing a point mutation (*srb10*-3) that inactivates the kinase function of SRB10 without affecting its stable incorporation into the holoenzyme. We infer that the defect is in transcriptional induction rather than in messenger RNA stability because mutations in *SRB10*/*UME5* do not destabilize vegetative mRNAs and actually increase meiotic mRNA stability twofold[13]. *SRB10* and *SRB11* mutant cells are slow growing, but control experiments show that slow growth does not inhibit induction by galactose (Fig. 3). The poor response of *srb10* and *srb11* mutant cells to transcriptional induction *in vivo* is consistent with the association of these two gene

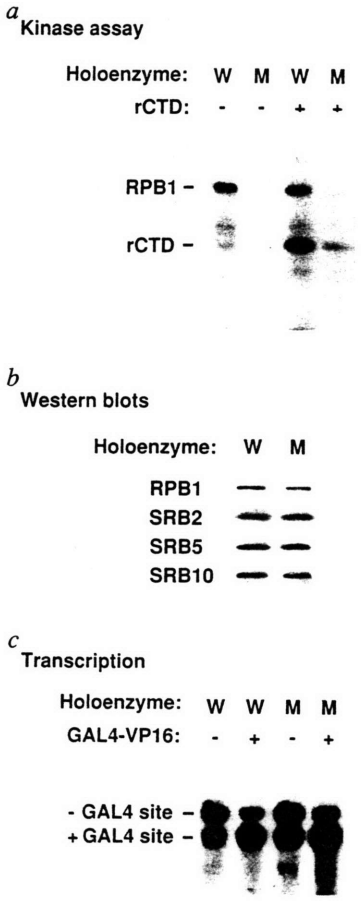

FIG. 4 CTD phosphorylation and transcription *in vitro* with wild-type and mutant RNA polymerase II holoenzymes. *a*, Purified holoenzyme lacking SRB10 function exhibits reduced CTD phosphorylation. Wild-type (W) and mutant (M) holoenzymes were assayed for their ability to phosphorylate the CTD of the RNA polymerase II large subunit or recombinant CTD added to the holoenzyme preparations. The mutant RNA polymerase II holoenzyme contains SRB10 protein in which Asp 290 has been replaced with Ala. Phosphorylation of the endogenous RNA polymerase II CTD was reduced ~10-fold and ~5-fold for recombinant CTD, in the mutant holoenzyme. *b*, Western blots showing that the two holoenzyme preparations contain similar amounts of the largest subunit of RNA polymerase II (RPB1) and SRB proteins. *c*, Wild-type and SRB10 mutant holoenzymes exhibit similar basal and activated transcription *in vitro*. Lanes: 1, wild-type holoenzyme (W); 2, wild-type holoenzyme + GAL4–VP16; 3, SRB10 mutant holoenzyme (M); 4, SRB10 mutant holoenzyme + GAL4–VP16. The large transcript is derived from the pGALΔ template, which lacks a GAL4 binding site, and the small transcript is from pGAL × 6, which contains six GAL4-binding sites. METHODS. RNA polymerase II holoenzymes were purified from wild-type and *srb10-3* mutant cells as described[1]. Kinase assays were carried out at 24 °C with 100 ng holoenzyme in 15 μl buffer containing 20 mM HEPES, pH 7.6, 8 mM $MgSO_4$, 2.5 mM EGTA, 5% glycerol, 2 mM DTT and a mixture of phosphatase inhibitors (1 mM NaN_3, 1 mM NaF, 0.4 mM $NaVO_3$, 0.4 mM $NaVO_4$ and 0.1 mg ml^{-1} phosvitin). Recombinant GST–CTD (100 ng) was added to a subset of the reactions; GST itself is not phosphorylated by holoenzyme preparations (not shown). Transcription was performed as described[29] with the following modifications: 100 ng pGALΔ and pGAL × 6 templates[30] were used per reaction; factors and DNA were preincubated for 15 min before adding nucleotides; and the stop mixture contained 12.5 U ml^{-1} ribonuclease T1. TFIIE was prepared as described[29] except that the second BioRex column was omitted and a Mono-S column step was added at the end.

products with CTD function, which was previously implicated in such responses.

The RNA polymerase II CTD is found in an unphosphorylated form in transcription initiation complexes but is extensively phosphorylated during elongation[11,23]. Thus, CTD phosphorylation may regulate some event in transcription initiation. We tested whether SRB10/11 kinase has a role in CTD phosphorylation in the holoenzyme using purified RNA polymerase II holoenzyme from wild-type and *srb10* mutant cells. Figure 4 shows that CTD phosphorylation was reduced ~10-fold in the *srb10* mutant holoenzyme, indicating that the SRB10 kinase must be important for CTD phosphorylation; the reduced response of SRB10-deficient holoenzyme to galactose induction *in vivo* may therefore reflect its diminished ability to phosphorylate the CTD. The yeast general transcription factor TFIIH is present in the holoenzyme[1], and the TFIIH-associated kinase[24,25] may account for the residual CTD phosphorylation in the mutant holoenzyme.

The activities of wild-type and SRB10-mutant RNA polymerase II holoenzymes were compared in a reconstituted transcription assay (Fig. 4*c*); levels of basal and GAL4–VP16-activated transcription were similar for both holoenzymes. We could not find any defect in transcription *in vitro* comparable to the loss of CTD phosphorylation *in vitro* or of galactose induction *in vivo*. These results suggest that factors necessary to elicit the regulatory role of SRB10 are missing or not functional in our *in vitro* transcription systems. Alternatively, the holoenzymes may contain additional kinases that compensate for the loss of SRB10 function in these systems.

We have identified a kinase-cyclin pair in the RNA polymerase II holoenzyme, shown that it is involved in transcriptional regulation *in vivo* and in CTD phosphorylation *in vitro*. Although the exact role of CTD phosphorylation in transcriptional regulation is not known, our results demonstrate that the response of the transcription initiation apparatus to at least some regulatory signals *in vivo* involves the SRB kinase–cyclin pair. □

Received 1 December 1994; accepted 20 January 1995.

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ACKNOWLEDGEMENTS. S.-M.L. and J.Z. contributed equally to this work. We thank A. Patel for technical assistance, S. Okamura, C. Hengartner and S. S. Koh for their contributions to the genetic and biochemical analysis, and G. Fink, T. Orr-Weaver and members of the Fink, Weinberg and Young laboratories for discussion. This research was supported by grants from the NIH (to R.A.Y.) and the Foundation for Research and Development (to H.J.J.v.V.). D.M.C. is a predoctoral fellow of the Howard Hughes Medical Institute.

Cell, Vol. 73, 1361–1375, July 2, 1993, Copyright © 1993 by Cell Press

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

Craig M. Thompson, Anthony J. Koleske, David M. Chao, and Richard A. Young
Whitehead Institute for Biomedical Research
Nine Cambridge Center
Cambridge, Massachusetts 02142
and Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

We report genetic and biochemical evidence that the RNA polymerase II carboxy-terminal domain (CTD) interacts with a large multisubunit complex that contains TATA-binding protein (TBP) and is an integral part of the transcription initiation complex. The isolation and characterization of extragenic suppressors of S. cerevisiae RNA polymerase II CTD truncation mutations led us to identify *SRB2*, *SRB4*, *SRB5*, and *SRB6* as genes involved in CTD function in vivo. *SRB2* was previously isolated and shown to encode a 23 kd TBP-binding protein. The four 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 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 by Sawadogo and Sentenac, 1990; Roeder, 1991; Sharp, 1991; Gill and Tijan, 1992; Zawel and Reinberg, 1992). One of these factors, transcription factor IID (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, TFIIE, TFIIF, 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 make up 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 eight 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 promoter selectivity factor, SL1, which contains TBP and three TAFs (Comai et al., 1992). A third complex is a component of the RNA polymerase III factor TFIIIB, which consists of TBP and two 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 by Corden, 1990; Young, 1991). The CTD contains 26–52 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., 1987b; 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., 1987b; 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 RNA polymerase–associated proteins (RAPs) (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/δ, TBP, and TFIIB/α (Peterson et al., 1990; Conaway and Conaway, 1991; Gerard et al., 1991; Conaway et al., 1991; Ha et al., 1991; Serizawa et al., 1992), 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 tran-

scription factor TFIIIB (Buratowski and Zhou, 1992; López-de-León et al., 1992; Kassavetis et al., 1992), but no yeast counterparts 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 homolog of the mammalian factor TFIIB (Pinto et al., 1992). TDS4/PCF4/BRF1 is related to TFIIB and is a component of TFIIIB (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; López-de-León 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 CTD truncation mutations, *SRB2-1* (SRB, suppressor of RNA polymerase B), 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. Furthermore, the identification of a putative yeast TAF as a suppressor of an RNA polymerase II CTD truncation suggests that the CTD might be used to identify additional TBP-associated components of the RNA polymerase II transcription initiation complex in yeast.

We report here genetic and biochemical evidence that the yeast RNA polymerase II CTD interacts with a large multisubunit complex that contains TBP. A genetic selection was used to isolate and characterize genes encoding proteins that influence CTD function. Antibodies generated against recombinant proteins encoded by four of these *SRB* genes were used to identify and purify a high molecular weight complex that 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

Suppressors of RNA Polymerase II CTD Truncation Mutations

To identify components of the transcription apparatus that affect CTD function, extragenic suppressors of a Saccharomyces cerevisiae RNA polymerase II CTD truncation mutant were isolated. The cold-sensitive phenotype of cells containing RNA polymerase II CTDs with only 11 intact heptapeptide repeats (*rpb1Δ104*) was exploited to obtain 85 independent suppressing isolates, of which approximately one-third were dominant and two-thirds recessive. The dominant suppressing isolates were chosen for further study. Genetic analysis revealed that all of the dominant mutations occurred in four *SRB* genes: *SRB2*, *SRB4*, *SRB5*, and *SRB6* (C. M. T. and J. Zhang, unpublished data). Additional analysis revealed that *SRB4*, *SRB5*, and *SRB6* are newly identified genes, whereas *SRB2* had previously been isolated in a similar genetic selection (Nonet and Young, 1989).

Two genetic assays were performed to obtain support for a functional relationship between the SRB gene products and the CTD (Figure 1). The ability of the suppressing alleles of *SRB4*, *SRB5*, and *SRB6* to suppress all of the phenotypes associated with the CTD truncation mutation *rpb1Δ104* was investigated. These phenotypes include cold- and temperature-sensitive growth, inositol auxotrophy, and the inability to utilize pyruvate as a carbon source. Cells containing either *SRB4-1*, *SRB5-1*, or *SRB6-1* suppress all of these defective phenotypes (Figure 1B), 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, we investigated the ability of the *SRB* alleles to suppress the conditional phenotypes associated with mutations elsewhere in RNA polymerase II (Figure 1C). *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 this 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*

We isolated genomic DNA clones containing *SRB4-1*, *SRB5-1*, and *SRB6-1* by taking advantage of their ability to suppress dominantly 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$^+$ transformants able to grow at 12°C. The mutant genes were further localized by constructing subgenomic libraries with fragments of the *SRB4-1*, *SRB5-1*, and *SRB6-1* genomic inserts and again selecting for Ura$^+$ transformants able to grow at 12°C. 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 did 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 λ 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 (λ clones 5961 and 6224). *SRB5* maps to the right arm of chromosome VII, approximately 30 kb centromere proximal to *SPT6* (λ clones 5146 and 4624). *SRB6* maps

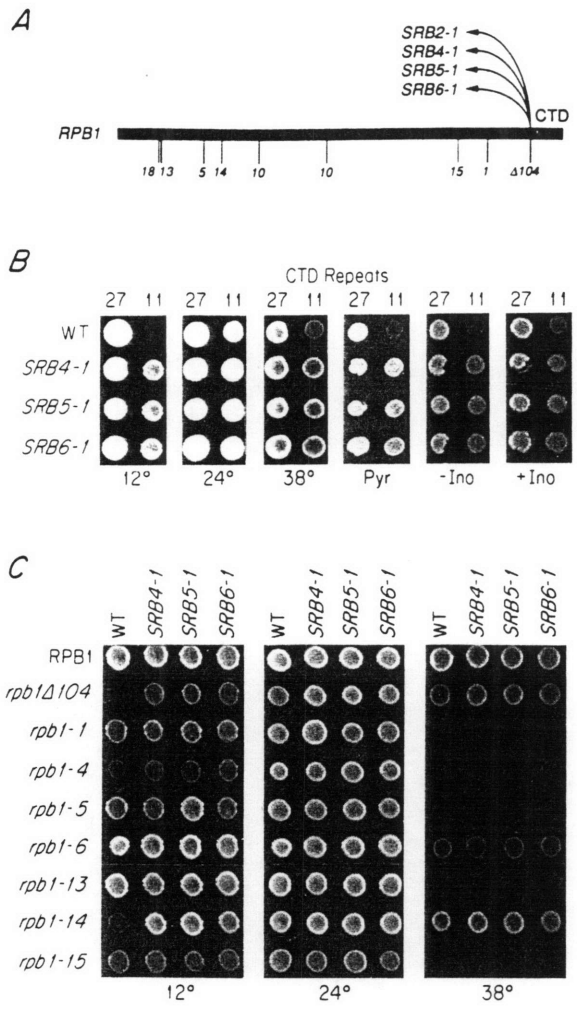

Figure 1. Genetic Characterization of *SRB4-1*, *SRB5-1*, and *SRB6-1*

(A) Diagram of *RPB1* conditional mutations used to isolate and characterize suppressors of *rpb1Δ104* mutations. The positions of the conditional mutations utilized in this study are indicated, except for those of *rpb1-4*, *rpb1-6*, and *rpb1-12*, 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 (panels 1–3), on SC medium containing pyruvate as a sole carbon source (panel 4), and on minimal medium with or without inositol (panels 5 and 6). Isogenic wild-type, *SRB4-1*, *SRB5-1* and *SRB6-1* backgrounds contained either wild-type *RPB1* (27 repeat CTD) or *rpb1Δ104* (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*.

to the right arm of chromosome II, approximately 75 kb centromere distal to *CDC28* (λ 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 amino acids long and has a molecular mass of 78 kd (Figure 2A). SRB5 is predicted to be 307 amino acids in length with a molecular mass of 34 kd (Figure 2B). The predicted SRB6 protein is 121 amino acids long and has a molecular mass of 14 kd (Figure 2C). A search of sequence data banks revealed that SRB4, SRB5, and SRB6 do not have significant sequence similarity to previously identified proteins. One notable feature of the SRB proteins is their acidic content. The predicted pK_a values of SRB2, SRB4, SRB5, and SRB6 are 5.2, 5.1, 4.7, and 4.6, respectively.

The suppressing mutations in all three genes were iden-

A SRB4

```
  1  MTTEDPDSNHLSSETGIKLALDPNLITLALSSNPN
 36  SSLHSPTSDEPVPESAGKADTSIRLEGDELENKTK
 71  KDNDKNLKFLKNKDSLVSNPHEIYGSMPLEQLIPI
106  ILRQRGPGFKFVDLNEKELQNEIKQLGSDSSDGHN
141  SEKKDTDGADENVQIGEDFMEVDYEDKDNPVDSRN
176  ETDHKTNENGETDDNIETVMTQEQFVKRRRDMLEH
211  INLAMNESSLALEFVSLLLSSVKESTGMSSMSPFL
246  RKVVKPSSLNSDKIPYVAPTKKEYIELDILNKGWK
281  LQSLNESKDLLRASFNKLSSILQNEHDYWNKIMQS
316  ISNKDVIFKIRDRTSGQKLLAIKYGYEDSGSTYKH
351  DRGIANIRNNIESQNLDLIPHSSSVFKGTDFVHSV
386  KKFLRVRIFTKIESEDDYILSGESVMDRDSESEEA
421  ETKDIRKQIQLLKKIIFEKELMYQIKKECALLISY
456  GVSIENENKVIIELPNEKFEIELLSLDDDSIVNHE
491  QDLPKINDKRANLMLVMLRLLLVVIFKKTLRSRIS
526  SPHGLINLNVDDDILIIRPILGKVRFANYKLLLKK
561  IIKDYVLDIVPGSSITETEVEREQPQENKNIDDEN
596  ITKLNKEIRAFDKLLNIPRRELKINLPLTEHKSPN
631  LSLMLESPNYCNALIHIKFSAGTEANAVSPDTTFS
666  DFKEVEDFLHFIVAEYIQQKKV  687
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B SRB5

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  1  MVQQLSLFGSIGDDGYDLLISTLTTISGNPPLLYN
 36  SLCTVWKPNPSYDVENVNSRNQLVEPNRIKLSKEV
 71  PFSYLIDETMMDKPLNFRILKSFTNDKIPLNYAMT
106  RNILHNTVPQVTNFNSTNEDQNNSKHTEDTVNESR
141  NSDDIIDVDMDASPAPSNESCSPWSLQISDIPAAG
176  NNRSVSMQTIAETIILSSAGKNSSVSSLMNGLGYV
211  FEFQYLTIGVKFFMKHGLILELQKIWQIEEAGNSQ
246  ITSGGFLLKAYINVSRGTDIDRINYTETALMNLKK
281  ELQGYIELSVPDRQSMDSRVAHGNILI  307
```

C SRB6

```
  1  MSNQALYEKLEQTRTILSVKLAELINMTTIADRND
 36  DDEGSFAQENSELAVATTSVMMVNNQTMQLIKNVQ
 71  DLLILTRSIKEKWLLNQIPVTEHSKVTRFDEKQIE
106  ELLDNCIETFVAEKTT  121
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Figure 2. Predicted Amino Acid Sequences of SRB4, SRB5, and SRB6 Proteins

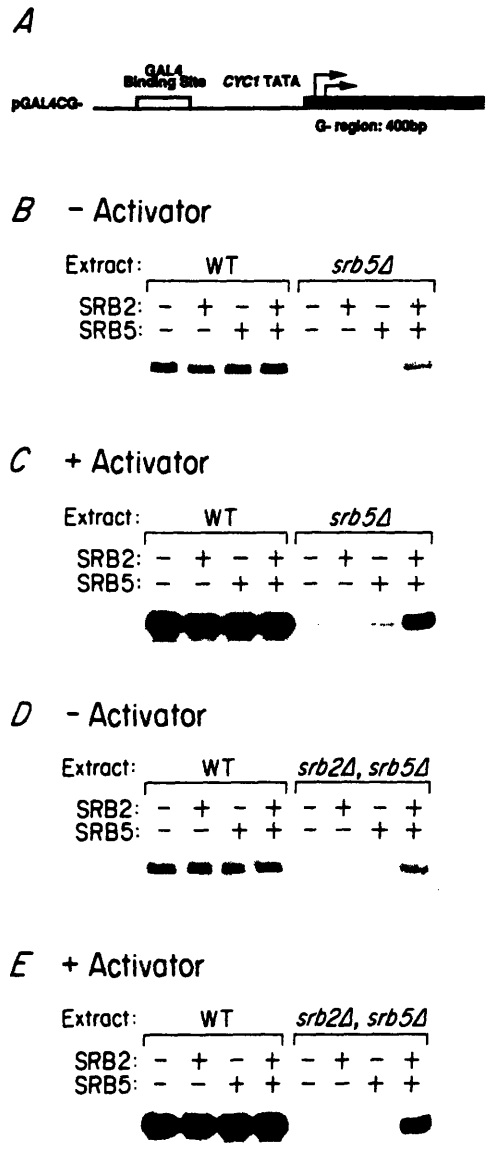

Figure 3. SRB2 and SRB5 Are Required for Efficient Transcription In Vitro

(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 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 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.

tified 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 *srb4Δ2*, *srb5Δ1*, and *srb6Δ1*. *SRB4* and *SRB6* are essential. *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.

SRB2 and SRB5 Are Required for Efficient Transcription In Vitro

Although yeast cells lacking *SRB4* or *SRB6* are not viable, cells lacking *SRB2* or *SRB5* are viable despite striking defects in growth, and it is this feature that facilitates investigation of the transcriptional activity of SRB2 and SRB5 proteins using nuclear extracts in vitro. Previous studies had 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 3). 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. 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, in both the presence and the absence of GAL4–VP16 (Figures 3B and 3C). 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 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 (Figures 3D and 3E). 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 (data not shown). These results indicate that both SRB2 and SRB5 are required for efficient basal and activated transcription initiation in vitro. If activated transcription is simply a stimulation of the basal process, then

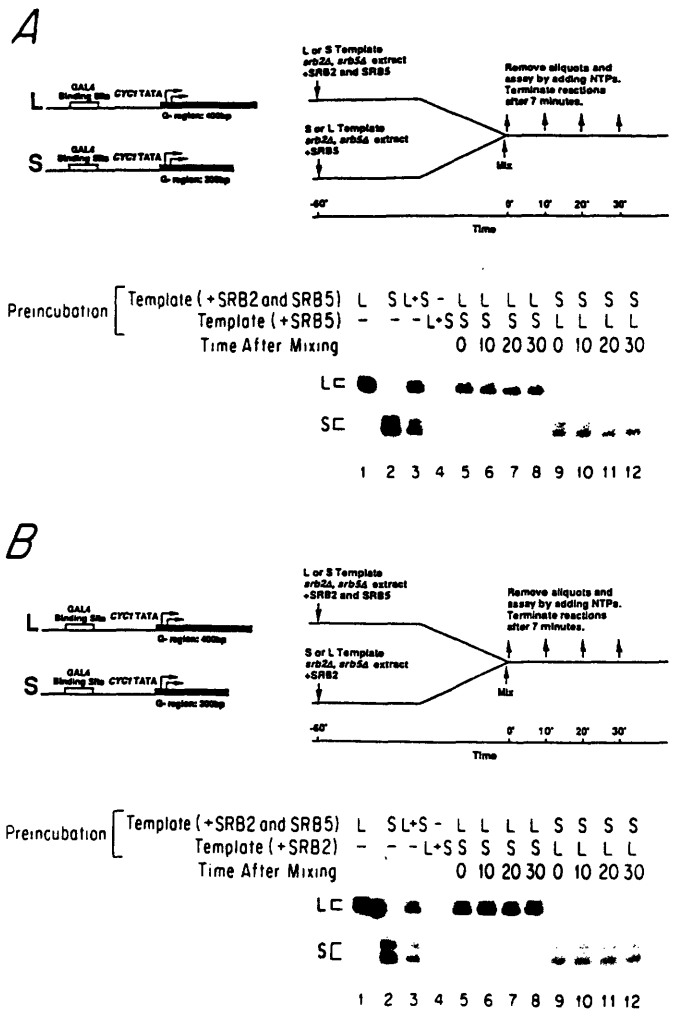

Figure 4. SRB2 and SRB5 Are Essential for Efficient Preinitiation Complex Formation

(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 1 of the 2 reaction mixtures. After a 60 min preincubation, the 2 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 short and long 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 that preincubations were performed in the presence or absence of limiting amounts of SRB5 (75 ng) and excess of SRB2 (250 ng).

SRB2 and SRB5 can be considered basal transcription factors. However, if the mechanism of transcriptional activation is more complex, then these SRB proteins may have additional roles in activated transcription.

Formation of a Stable Preinitiation Complex Involves SRB2 and SRB5

A template commitment assay was used to investigate whether both SRB2 and SRB5 participate in the formation of a transcription initiation complex (Figure 4). Extracts prepared from cells lacking SRB2 and SRB5 were used for performing this assay. Two templates were employed that contained identical promoters but differed in G-less cassette length. Specific transcripts of 375 and 350 nt were produced from the long template, while transcripts of 275 and 250 nt were produced from the short template.

We first performed an experiment to confirm that SRB2 is required for efficient formation of a stable preinitiation complex (Figure 4A), 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 1 of the 2 reaction mixtures. After a 60 min preincubation, the 2 reactions were mixed together. Immediately after mixing and every 10 min thereafter, aliquots were removed and nucleoside triphosphates were added to permit RNA synthesis. The reaction was stopped after 7 min to minimize multiple rounds of transcription. Control experiments are shown in lanes 1–4. When *srb2Δ1*, *srb5Δ1* extracts were preincubated with SRB2 and SRB5 along with either the long template (Figure 4A, lane 1) or short template (Figure 4A, 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 (Figure 4A, lane 3). Virtually no transcript was detected when both templates were preincubated with the extract in the presence of SRB5 alone (Figure 4A, lane 4). When SRB2 was added to the long template mixture, long transcripts were predominant after the two extracts were mixed (Figure 4A, 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 (Figure 4A, lanes 9–12).

To determine whether SRB5 is required for efficient preinitiation complex formation, a similar experiment was performed (Figure 4B). This time, the two templates were incubated separately with extract and SRB2, and a limiting amount of SRB5 was added to 1 of the 2 reaction mixtures. The remaining steps were performed as described above. The results of the controls (Figure 4B, lanes 1–4) were identical to those in Figure 4A. Lanes 5–12 in Figure 4B 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 was preferentially transcribed, upon mixing, relative to the other template, which was incubated in the absence of either SRB2 or SRB5. Second, following mixing, there was 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; since up to 30 min of incubation was allowed after template mixing, there was ample time for any catalytic activity to be carried out on the second template. The observation of little to no increase in second template transcription, even after 30 min, indicates that SRB2 and SRB5 became stably associated with the first template during preincubation.

When the experiment in Figure 4A was performed using excess SRB2 in the preinitiation step, transcription increased with time from the template that was preincubated in the absence of SRB2 (data not shown). Similarly, when the experiment in Figure 4B was performed using excess SRB5 in the preincubation step, transcription increased with time from the template that was preincubated in the absence of SRB5 (data not shown). This indicates that much of the template that was preincubated in the absence of SRB2 or SRB5 was still available for transcription and that SRB2 and SRB5 continued 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.

SRB Proteins, TBP, and RNA Polymerase 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 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 data). 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 and against recombinant TBP were used to monitor these proteins during purification (Figure 5). Essentially all of the SRB2, SRB4, SRB5, and SRB6 in the whole-cell extract cofractionated through the seven purification steps. Approximately 20 additional polypeptides, including a portion of the TBP in 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 appeared to be quite stable. The proteins in this complex remained 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 5C shows, for example, the elution profile of TBP, SRB proteins, and RNA polymerase II from the Mono S column. We estimated that the complex was purified approximately 10,000-fold by quantitative Western blot analysis. The complex appeared 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 6 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 (not shown).

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 in-

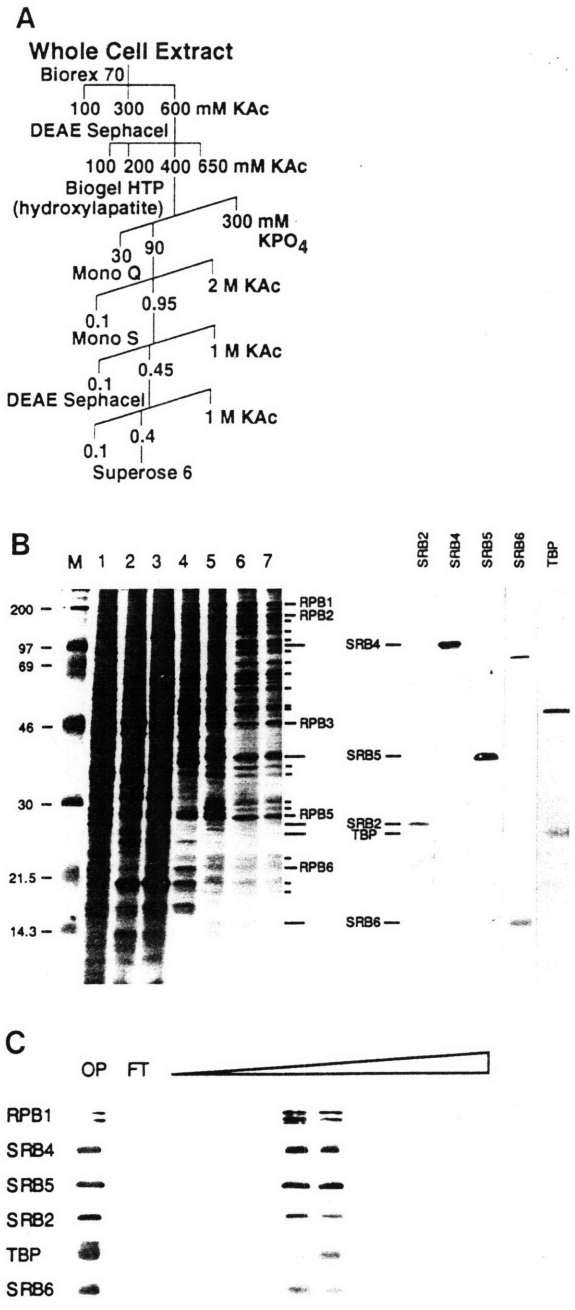

Figure 5. Purification of the SRB Complex

(A) Fractionation scheme.

(B) The left panel shows a silver-stained SDS–polyacrylamide (15%) gel containing approximately 1 μg of protein from each fraction of the SRB complex purification. 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. M, markers.

The right panel shows Western blot analysis of 1 μg of SRB complex protein from the DEAE–Sephacel fraction loaded onto a SDS–polyacrylamide (15%) 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; and 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 of total protein) from the Mono Q column was loaded onto a Mono S column and eluted with a 0.1–1.0 M gradient of potassium acetate as described in Experimental Procedures. The onput and flow-through material (1/25) and every other eluate fraction (1/50) 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.

vestigate this possibility, yeast whole-cell extract was loaded onto columns containing recombinant glutathione S-transferase (GST)–CTD fusion protein or GST alone, the columns were washed extensively, and bound protein was eluted with low concentrations of guanidine hydrochloride (Figure 7). 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 in lacking 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.

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. The isolation and genetic characterization of four dominant extragenic suppressors of RNA polymerase II CTD truncation mutations indicated that at least four *SRB* gene products are involved in the same function as the RNA polymerase II CTD. Large multisubunit complexes containing the four 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.

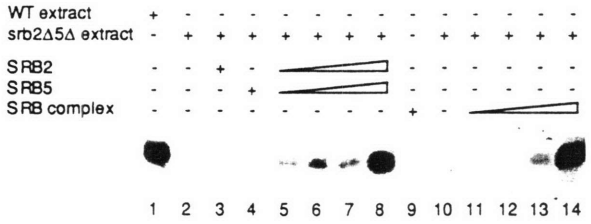

Figure 6. The Purified Complex Contains SRB2 and SRB5 Activities

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 of SRB2; lane 4, 500 ng of 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, 500 ng 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 microgram of SRB complex contained approximately 20 ng of SRB2 and 25 ng of SRB5, as estimated by quantitative Western analysis with known amounts of recombinant SRB2 and SRB5 proteins.

SRB Suppressors of CTD Mutations

The *SRB2* gene was previously isolated as a dominant, gain of function suppressor of RNA polymerase II CTD truncation mutations and was subsequently shown to encode a TBP-associated factor (Koleske et al., 1992). Reasoning that additional extragenic suppressors might be obtained that could encode TAFs or other components of the transcription initiation complex, we isolated approximately 30 dominant suppressing isolates of CTD truncation mutations, and found that all occur in *SRB2*, *SRB4*, *SRB5*, and *SRB6*. Mutations in these four *SRB* genes specifically suppress CTD truncation mutations, indicating that the CTD and the *SRB* gene products are involved in the same functional process during initiation.

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, the SRB proteins, and TBP cofractionated with additional proteins through seven chromatographic purification steps. The purified 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 two dozen protein components of the complex, which add up to 1.4 Md, assuming a subunit stoichiometry of 1. 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 acitivity 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 (A. J. K., unpublished data). 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. About 5%–10% of total cellular TBP is associated with the high molecular weight complex. These data suggest that transcription initiation complexes containing the SRB proteins occur at approximately 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

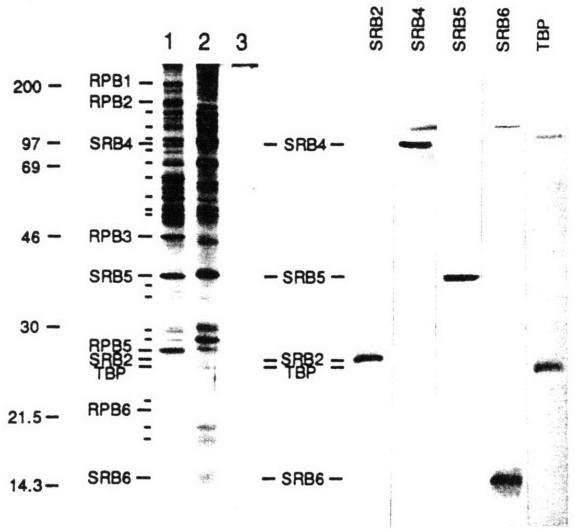

Figure 7. A TBP-Associated Complex Binds to the RNA Polymerase II CTD

(Left panel) Silver-stained SDS–polyacrylamide (15%) 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.

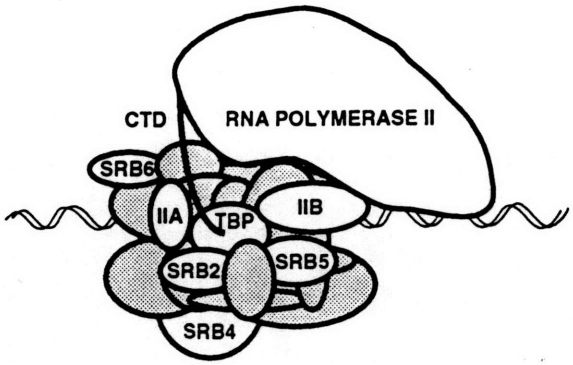

Figure 8. Model of Preinitiation Complex Containing the SRB–TBP–RNA Polymerase II Complex

The diagram is drawn to emphasize physical relationships between the RNA polymerase II CTD and TBP and between TBP and SRB2 and SRB5. The nomenclature for general transcription factors from higher eukaryotes is used. The relative positions of RNA polymerase II, TBP, TFIIA, and TFIIB are based upon DNAase I footprint analysis (Buratowski et al., 1989). Other aspects of the arrangement of the proteins in the complex are purely hypothetical. It is not clear whether TFIIA and TFIIB are components of the purified SRB–TBP–RNA polymerase II complex.

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 the 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(τ) 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 homologs of mammalian and Drosophila TAFs? TAFs are not yet well defined, and at least two criteria have been used to describe them. TAFs can be described simply as proteins tightly associated with TBP. Using this definition, SRB proteins are yeast TAFs. Some mammalian and Drosophila TAFs 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 TAFs 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 with the criteria attributed to coactivators that 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 that indicates that it is physiologically significant. However, we have only limited clues to the structure of the complex. Thus far, the evidence indicates that the RNA polymerase II CTD interacts with TBP which, in turn, is associated with a complex of proteins that include SRBs (Figure 8). SRB2 and SRB5 can bind directly to TBP (Koleske et al., 1992; C. M. T., unpublished data). Other components of this complex may contact these SRB proteins, TBP, and/or RNA polymerase II. It is not yet clear whether the complex we have purified assembles independently of promoter DNA in vivo or has dissociated from promoters upon cell disruption. Indeed, it is possible that the SRBs are brought to the initiation complex in association with RNA polymerase II independently of TBP.

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 maps to the upstream activating sequences (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 that are 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 sig-

Table 1. Yeast Strains

Strain	Alias	Genotype
BJ926		*Mat a/Mat α trp1/TRP1 prc1-126/prc1-126 pep4-3/pep4-3 prp1-1122/prb1-1122 can1/can1*
Z22	N114	*Mat α ura3-52 his3Δ200 leu2-3,112*
Z26	N247	*Mat α ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3* (pRP112[*URA3 RPB1*])
Z28	RY4	*Mat a/MAT α mal-/mal- gal2/gal2*
Z425	YTK73	*Mat a his3Δ200 leu2-3,112 ura3-52 trp1Δ1 lys2-801 srb2Δ1::HIS3*
Z551	N400	*Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3* (pC6[*LEU2 rpb1Δ104*])
Z552	CTY3	*Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 SRB4-1* (pC6 [*LEU2 rpb1Δ104*])
Z553	CTY8	*Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 SRB5-1* (pC6 [*LEU2 rpb1Δ104*])
Z554	CTY9	*Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 SRB6-1* (pC6 [*LEU2 rpb1Δ104*])
Z555	CTY15	*Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 SRB4-1* (pRP112 [*URA3 RPB1*])
Z556	CTY20	*Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 SRB5-1* (pRP112 [*URA3 RPB1*])
Z557	CTY21	*Mat a ura3-52 his3Δ200 leu2-3,112 rpb1Δ187::HIS3 SRB6-1* (pRP112 [*URA3 RPB1*])
Z558	CTY143	*Mat a/MAT α ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112*
Z559	CTY144	*Mat a/MAT α ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 srb5Δ1::URA3hisG/SRB5*
Z560	CTY148	*Mat α ura3-52 his3Δ200 leu2-3,112 srb5Δ1::URA3hisG*
Z561	CTY151	*Mat a ura3-52 his3Δ200 leu2-3,112 lys2-801*
Z562	CTY153	*Mat a ura3-52 his3Δ200 leu2-3,112 lys2-801 srb5Δ1::URA3hisG*
Z563	CTY154	*Mat a ura3-52 his3Δ200 leu2-3,112 lys2-801 srb2Δ1::HIS3 srb5Δ1::URA3hisG*
Z564	CTY158	*Mat a/Mat α ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 srb6Δ1::URA3hisG/SRB6*
Z565	CTY176	*Mat a/Mat α ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 srb4Δ2::HIS3/SRB4*
Z566	CTY184	*Mat a ura3-52 his3Δ200 leu2-3,112 srb6Δ1::hisG* (pCT66 [*LEU2 SRB6*])

nals. 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). Thus, the SRB–TBP complex may process multiple signals at promoters and govern the decision to initiate transcription.

Experimental Procedures

Genetic Manipulations

Yeast strains and plasmids are listed in Tables 1 and 2, respectively. Yeast medium was prepared as described (Nonet and Young, 1989), except pyruvate medium, which consists of synthetic complete (SC) medium with 2% pyruvic acid (Sigma) as a 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-fluoroorotic acid (5-FOA) as a selective agent against *URA3* plasmids.

Extragenic suppressors of the cold-sensitive phenotype of Z551 were isolated as previously described (Nonet and Young, 1989). Dominant 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. Isogenic wild-type, *SRB4-1*, *SRB5-1*, and *SRB6-1* strains containing various *RPB1* alleles (*rpb1-4*, *rpb1-5*, *rpb1-6*, *rpb1-10*, *rpb1-12*, *rpb1-13*, *rpb1-14*, *rpb1-15*, and *rpb1-18*) on *LEU2 CEN* plasmids (Scafe et al., 1990) 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 medium to permit loss of pC6 (Nonet et al., 1987b). Growth assays were performed by suspending similar numbers of cells in water and transferring equal volumes to agar plates with a 48 prong apparatus.

Deletions of *SRB4*, *SRB5*, and *SRB6* were created by a single step disruption method (Rothstein, 1991). Z558 was transformed with the desired DNA fragment and plated on the proper selective medium. Southern analysis was used to confirm that a single copy of the desired *SRB* gene had been deleted. The diploid was sporulated and tetrads (more than 20) were dissected 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 medium. Two spores or fewer from each tetrad were viable, and these 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^- Ura^+ segregants were streaked to 5-FOA plates. They were unable to grow on 5-FOA-containing medium, confirming that *SRB4* is essential. Z559 was created by transformation with the EcoRI–SphI fragment of pCT37 containing the *srb5Δ1::URA3-hisG* fragment and plating on SC–Ura medium. 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 medium. Two spores or fewer 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^+ Leu^+ 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 medium to permit loss of pCT66, and then tested for growth on 5-FOA plates. No growth was observed on 5-FOA, confirming that *SRB6* is essential.

Several strains were constructed for producing yeast nuclear extracts for in vitro transcription assays. Z425 was mated to Z560, and tetrads were dissected to produce the wild-type Z561, *srb5Δ1::URA3-hisG* strain Z562, and *srb2Δ1::HIS3, srb5Δ1::URA3hisG* strain Z563. Z562 and Z563 displayed 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). Polymerase chain reaction (PCR) amplifications to produce pCT54 (*srb4Δ2*), pCT37 (*srb5Δ1*), and pCT38 (*srb6Δ1*) were performed with Taq DNA polymerase (Perkin Elmer) in 100 μl of buffer (provided by the manufacturer) supplemented with 1.0 mM $MgCl_2$ 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

Table 2. Plasmids

Plasmid	Description
pCT3	*URA3 CEN* 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
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$^-$.
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–SalI–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.
SRB4	
pCT4	pCT3 with 9 kb genomic (Z28) Sau3A fragment containing *SRB4* inserted at XhoI site.
pCT8	pCT3 with 8 kb genomic (Z552) Sau3A fragment containing *SRB4-1* inserted at XhoI site.
pCT15	pCT3 with 2.5 kb subgenomic (pCT4) Sau3A fragment containing *SRB4* inserted at XhoI site.
pCT16	pCT3 with 2.8 kb subgenomic (pCT4) Sau3A fragment containing *SRB4* inserted at XhoI site.
pCT48	pCT15 with BstXI–SnaBI *SRB4-1* C-terminus fragment from pCT8 replacing same *SRB4* fragment.
pCT54	*srb4Δ2::HIS3*, created by ligation of *SRB4* SalI–BamHI from pCT16 with SalI–BamHI of pSP72 (Promega), followed by PCR with the oligonucleotides TAATATCCTGAGTCACTCCT and TATGGCTTTTAAGCTGCTTA and ligation of PCR product with SmaI *HIS3 kan* fragment from B2179 (G. R. Fink, Whitehead).
pCT107	pGEX-2T (Smith and Johnson, 1988) with *GST–SRB4* fusion. NdeI site at ATG of *SRB4* created by ligation of *SRB4* SalI–XbaI from pCT15 with SalI–XbaI of pBSIISK(−) (Stratagene), followed by site-directed mutagenesis. NdeI (partial/blunt)–SnaBI *SRB4* containing fragment was then ligated with BamHI (blunt)–digested pGEX-2T.
SRB5	
pCT14	pCT3 with 9 kb genomic (Z553) Sau3A fragment containing *SRB5-1* inserted at XhoI site.
pCT20	pCT3 with 1.9 kb subgenomic (pCT14) Sau3A fragment containing *SRB5-1* inserted at XhoI site.
pCT32	pCT20 with unique SacI site in insert, created by removal of NarI (blunt)–SacII (blunt) fragment from vector.
pCT37	*srb5Δ1::URA3hisG*, created by ligation of *SRB5-1* EcoRI–BamHI from pCT20 with EcoRI–BamHI of pSP72 (Promega), followed by PCR with the oligonucleotides TAATCATTGGCACCTGGGCA and CTTTTCTTCTTAATATGGAA and ligation of PCR product with BglII (blunt)–BamHI (blunt) *URA3 kan hisG* cassette from B2178 (G. R. Fink).
pCT39	pCT32 containing *SRB5*, obtained by gap repair of vector containing fragment of pCT32 SacI–XhoI digest.
pCT98	pET-3a (Studier and Moffat, 1986) with *SRB5*. NdeI site at ATG of *SRB5* created by ligation of *SRB5* EcoRI–BamHI from pCT39 with EcoRI–BamHI of pBSIISK(−) (Stratagene), followed by site-directed mutagenesis. NdeI–EcoRI (blunt) *SRB5*-containing fragment was then ligated with NdeI–BamHI (blunt)–digested pET-3a.
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 oligonucleotides TAAAAAGGCGGTATTTATCT and CATATAGTGCCTGGTTGCTC and ligation of PCR product with BglII (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 BalI–SphI digest.
pCT66	*LEU2 CEN* pUN105 (Elledge and Davis, 1988) with *SRB6*, created by ligation of *SRB6* BamHI (blunt)–SalI (blunt) from pCT40 with SmaI-digested pUN105.
pCT116	pGEX-2T (Smith and Johnson, 1988) with *GST–SRB6* fusion. NdeI site at ATG of *SRB6* created by ligation of *SRB6* SalI–XbaI from pCT40 with SalI–XbaI of pBSIISK(+) (Stratagene), followed by site-directed mutagenesis. NdeI (blunt)–XbaI *SRB6*-containing fragment was then ligated with BamHI (blunt)–digested pGEX-2T.

DNA was isolated as described by Phillippsen et al. (1991), partially digested with Sau3A, and separated on a 0.7% agarose gel, 8–12 kb fragments were purified by electroelution, and the ends were partially filled in with d(AG)TP using Klenow. The *URA3* centromeric plasmid pCT3 was digested with XhoI, and the ends were partially filled in with d(CT)TP to make them compatible with the ends of the Sau3A–digested genomic DNA. Following ligation, DH5α 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 and separated on a 1.5% agarose gel, and 1–3 kb fragments were purified by gene clean (BIO 101), and the ends were partially filled in with d(AG)TP using Klenow. Fragments were ligated with pCT3 prepared as described above and transformed into DH5α 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 medium and placing plates at 12°C following a 12 hr recovery period at 30°C. Approximately 1 in 2000 primary transformants was able to grow at 12°C. For each library transformed, the genomic clone was isolated by the method of Hoffman and Winston (1987), from over 12 Ura$^+$ colonies able to grow at 12°C, and was 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 tight temperature-sensitive phenotype in combination with a CTD truncation allele of 11 repeats. The presence of pCT4 restores a leaky temperature-sensitive phenotype to this strain at 38°C. Subgenomic clones made from pCT4 (*SRB4*), pCT14 (*SRB5-1*), and pCT26 (*SRB6-1*) were selected as described above 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*. pCT39 was created from pCT32 in vivo by transforming Z22 with SacI–XhoI-digested pCT32 DNA and isolating the plasmid from a Ura$^+$ transformant that 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.

Sequence Analysis

Insert DNAs from pCT15, pCT20, and pCT29 (containing *SRB4*, *SRB5-1*, and *SRB6-1*, respectively) were completely sequenced on each strand. 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. The suppressing mutations in *SRB4*, *SRB5*, and *SRB6* were deduced by sequencing using oligonucleotide primers that spanned the entire open reading frames. Positive numbering of the DNA begins with the predicted start site of translation. pCT15 (*SRB4*) and pCT48 (*SRB4-1*) were sequenced, and the *SRB4-1* mutation was identified as a G to T transversion (nucleotide 1057) that changed amino acid 353 from Gly to Cys. pCT39 (*SRB5*) and pCT32 (*SRB5-1*) were sequenced, and the *SRB5-1* mutation was identified as a C to T transition (nucleotide 65) that changed amino acid 22 from Thr to Ile. pCT40 (*SRB6*) and pCT29 (*SRB6-1*) were sequenced, and the *SRB6-1* mutation was identified as a C to G transversion (nucleotide 258) that changed amino acid 86 from Asn to Lys. Sequence comparison analysis was performed at the National Center for Biotechnology Information using the BLAST network service (Altschul et al., 1990).

Purification of Recombinant Proteins

Purification of SRB2 has been previously described (Koleske et al., 1992). SRB5 protein was purified from the bacterial strain BL21(DE3) pLysS (Studier and Moffatt, 1986) carrying the plasmid pCT98 in the same manner in which SRB2 was purified. SRB4 and SRB6 were purified as fusions to GST from DH5α carrying pCT107 and pCT116, respectively, according to the 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 DH5α 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 was carried out as described by Liao et al. (1991). Three hundred nanograms of template were used for promoter-dependent in vitro transcription reactions, except the template commitment assays, in which 600 ng of template was used per reaction. Optimal activity was obtained using 100 μg of Z561 protein, 150 μg of Z562 protein, and 150 μg of Z563 protein. Transcripts were quantified using a Fuji Bio-image analyzer. Promoter-independent transcription assays were performed according to Nonet et al. (1987b). 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) (buffer A containing 50 mM potassium acetate) and concentrated 4-fold by centrifugation through Centricon 10 filter units (Amicon).

Purification of SRB Complex

Yeast strain BJ926 (Buchman et al., 1988) was grown at 30°C to OD_{600} of 4.0–4.5 in 1 × YNB medium (0.15% Difco yeast nitrogen base, 0.5% ammonium sulfate, 200 μM inositol, 2% glucose). The level of the SRB complex appeared to be elevated in cells grown in minimal medium (A. J. K., unpublished data), 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 dithiothreitol [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 phenylmethylsulfonyl fluoride, 2 mM benzamidine, 2 μM pepstatin A, 0.6 μM leupeptin, 2 μg/ml chymostatin, 5 μg/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 hr, and the impregnation with silver nitrate was performed for 40 min.

Whole-cell extract (8 g of protein in 390 ml) was diluted 1:5 in buffer A (20% glycerol, 20 mM HEPES KOH [pH 7.5], 1 mM DTT, 1 mM EDTA, and protease inhibitors). The extract was loaded onto a 5 cm × 17 cm Biorex 70 (Bio-Rad Laboratories) column at a flow rate of 5 ml/min. The column was washed with buffer A (100) 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], 1 mM DTT, 1 mM EDTA, 0.01% Nonidet P-40, and protease inhibitors) and was loaded onto a 2.5 cm × 8.5 cm diethylaminoethyl (DEAE)–Sephacel column (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% Nonidet P-40, and protease inhibitors). The dialysate was spun in a Sorvall SS34 rotor at 10,000 rpm for 20 min and the supernatant (50 mg of protein in 50 ml) was loaded onto a 1.5 cm × 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 68–112 mM potassium phosphate.

The 20 ml of eluate from the Bio-Gel HTP (Bio-Rad Laboratories) 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 fast protein liquid chromatography 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 of protein in 10 ml) 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 of protein in 8 ml) was diluted 1:4 in buffer E (0) and loaded onto a 1.5 cm × 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 of protein in 2 ml) was diluted 1:4 in buffer F (0) and loaded onto a 1.5 cm × 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 and 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), and carbonic anhydrase (29 kd).

CTD Affinity Purification

Whole-cell extracts were prepared by adding 1.6 l of 4% glucose to 800 g of Red Star dry yeast, incubating the mixture at room temperature for 45 min, and adding 800 ml of disruption buffer (1.2 M ammonium sulfate, 0.16M K–HEPES [pH 7.3], 4 mM DTT, and protease inhibitors [as in the conventional purification above]). Aliquots (200 ml) were frozen dropwise in liquid nitrogen and blended for 5–10 min in a Waring

blender. After thawing at 55°C, viscosity was reduced by brief blending. Disrupted cells were centrifuged for 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 Polymin P was added, the extract was incubated on ice for 30 min, and the solution was centrifuged for 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 min. The pellet was resuspended in 1.5 vol of 1 × transcription buffer (Liao et al., 1991) plus protease inhibitors and centrifuged at 17,000 rpm in a Sorvall SS34 rotor for 20 min. The supernatant was then diluted 1:6 in 1 × transcription buffer plus protease inhibitors and centrifuged at 12,000 rpm in a Sorvall GSA rotor for 30 min. The supernatant was incubated with 10 g/100 milliliters 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 1–2 hr.

GST fusion proteins were coupled to Pharmacia activated CH Sepharose according to the manufacturer's directions at a concentration of 5 milligrams of protein per milliliter of matrix. The affinity matrices were washed with 6 M guanidine hydrochloride followed by 1 × transcription buffer before use. Twenty milliliters of yeast whole-cell extract were mixed with 1/10 vol of 1 × transcription buffer plus 10% Triton X-100 and applied to 100 μl of either GST–Sepharose or GST–CTD Sepharose. The columns were washed with 20 ml of 1 × transcription buffer plus 1% Triton X-100, followed by 5 ml of 1 × transcription buffer without Triton X-100. Bound proteins were eluted with 1 × transcription buffer containing various concentrations of guanidine hydrochloride.

Western Blot Analysis

Western blotting of fractions was performed with polyclonal rabbit antisera raised against whole TBP, SRB2, and SRB5 proteins, a GST–SRB4 fusion protein, or a GST–SRB6 fusion protein, by standard methods (Harlow and Lane, 1989). RPB1 was detected via the CTD with 8WG16 monoclonal antibody ascites fluid (Thompson et al., 1989). Polyclonal anti-TBP, anti-SRB2, anti-GST–SRB4, and anti-SRB5 antisera were diluted 1:1000. Anti-GST–SRB6 antiserum was diluted 1:200. A 1:1000 dilution of 8WG16 monoclonal antibody ascites fluid was used. In all cases, bands were visualized by secondary probing with alkaline phosphatase conjugate secondary antibodies (Promega).

Acknowledgments

We thank Sara Okamura and Jianhua Zhang for their contributions to the CTD suppressor selection and genetic analysis, Bill Jackson for identifying the *SRB4-1* mutation, Steve Buratowski for advice and anti-TBP antibodies, and Michael Sayre and Yang Li for helpful comments and suggestions. D. M. C. is a predoctoral fellow of the Howard Hughes Medical Institute. This work was supported by a grant from the National Institutes of Health.

Received January 7, 1993; revised April 14, 1993.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are L12026 (for *SRB4*), L12028 (for *SRB5*), and L12027 (for *SRB6*).

Cell, Vol. 84, 235–244, January 26, 1996, Copyright ©1996 by Cell Press

RNA Polymerase II Holoenzyme Contains SWI/SNF Regulators Involved in Chromatin Remodeling

Christopher J. Wilson,* David M. Chao,*
Anthony N. Imbalzano,† Gavin R. Schnitzler,†
Robert E. Kingston,† and Richard A. Young*
*Whitehead Institute for Biomedical Research
Nine Cambridge Center
Cambridge, Massachusetts 02142
and Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139
†Department of Molecular Biology
Massachusetts General Hospital
Boston, Massachusetts 02114
and Department of Genetics
Harvard Medical School
Boston, Massachusetts 02115

Summary

The RNA polymerase II holoenzyme contains RNA polymerase II, a subset of general transcription factors and SRB regulatory proteins. We report here that *SWI* and *SNF* gene products, previously identified as global gene regulators whose functions include remodeling chromatin, are also integral components of the yeast RNA polymerase II holoenzyme. The SWI/SNF proteins are components of the SRB complex, also known as the mediator, which is tightly associated with the RNA polymerase II C-terminal repeat domain. The SWI/SNF components provide the holoenzyme with the capacity to disrupt nucleosomal DNA and thus facilitate stable binding of various components of the transcription initiation complex at promoters.

Introduction

Regulation of class II genes involves a complex interplay among gene-specific activators and cofactors, the general transcription apparatus, and chromatin. Gene-specific activators bind to promoters and stimulate transcription, at least in part, by binding and recruiting the general transcription apparatus (Chen et al., 1994; Hengartner et al., 1995; Ingles et al., 1991; Lin et al., 1991; Xiao et al., 1994; reviewed by Tjian and Maniatis, 1994; Sheldon and Reinberg, 1995; Emili and Ingles, 1995; Carey, 1995). Chromatin structure can affect the transcriptional activity of genes by blocking access of the transcription apparatus to promoters (Knezetic and Luse, 1986; Bresnick and Felsenfeld, 1993; Felsenfeld, 1992; Lorch et al., 1988; Workman and Roeder, 1987). The SWI and SNF proteins are global regulators that function by antagonizing repression mediated by nucleosomes, altering chromatin structure to facilitate binding of the transcription apparatus (Côté et al., 1994; Hirschhorn et al., 1992; Imbalzano et al., 1994; Kwon et al., 1994; reviewed by Carlson and Laurent, 1994; Peterson and Tamkun, 1995; Winston and Carlson, 1992). It is not yet clear how the SWI/SNF proteins are targeted to promoters, although some gene-specific activators may interact directly with these proteins (Yoshinaga et al., 1992).

Genetic and biochemical studies in yeast indicate that the form of the transcription initiation apparatus generally responsible for mRNA synthesis in vivo is an RNA polymerase II holoenzyme (Barberis et al., 1995; Hengartner et al., 1995; Kim et al., 1994; Koleske and Young, 1994; Thompson and Young, 1995; reviewed by Carey, 1995; Emili and Ingles, 1995; Koleske and Young, 1995). This megadalton-sized complex contains RNA polymerase II, general transcription factors, and additional components called suppressor of RNA polymerase B (SRB) regulatory proteins. The SRB proteins are a hallmark of the holoenzyme. The genes encoding the nine known SRB proteins were identified through a selection for factors involved in transcription initiation by RNA polymerase II in vivo, and all are required for normal yeast cell growth. Essentially all of the SRB protein in cells is tightly associated with the holoenzyme, while approximately 80% of RNA polymerase II and general transcription factors are found independent of this complex (Koleske and Young, 1995). Experiments with temperature-sensitive *SRB* mutants indicate that the RNA polymerase II holoenzyme is the form of the transcription initiation apparatus employed at the majority of class II promoters in vivo (Thompson and Young, 1995). Other experiments have shown that recruiting a component of the SRB complex to promoters, presumably in association with the holoenzyme, suffices to obtain activated levels of transcription in vivo (Barberis et al., 1995).

The yeast *SWI* genes were first identified as positive regulators of HO transcription (Stern et al., 1984), and *SWI1*, *SWI2*, and *SWI3* were later shown to be required for the activation of a broad spectrum of inducible genes in vivo (Peterson and Herskowitz, 1992; Yoshinaga et al., 1992). Similarly, the *SNF* genes were originally identified as positive regulators of *SUC2* (Neigeborn and Carlson, 1984), and *SNF2*, *SNF5*, and *SNF6* were subsequently found to be essential for activation of a diverse set of inducible genes (Laurent and Carlson, 1992; Laurent et al., 1991; Peterson and Herskowitz, 1992). Further study revealed that *SWI2* and *SNF2* are the same gene. Genetic evidence indicated that the *SWI* and *SNF* genes are involved in similar processes in gene activation (Carlson and Winston, 1992). Indeed, the discovery that SWI1, SWI2/SNF2, SWI3, SNF5, SNF6, and SNF11 proteins copurify in a large complex confirmed that the *SWI/SNF* gene products function together (Cairns et al., 1994; Côté et al., 1994; Peterson et al., 1994; Treich et al., 1995). Genetic and biochemical evidence implicated the SWI/SNF proteins in chromatin remodeling via nucleosome disruption (Cairns et al., 1994; Côte et al., 1994; Hirschhorn et al., 1992; Peterson et al., 1994).

Several lines of evidence led us to investigate whether SWI and SNF proteins are components of the RNA polymerase II holoenzyme, and furthermore, whether SWI/SNF proteins are components of the SRB-containing protein complex that is tightly associated with the C-terminal repeat domain (CTD) in the holoenzyme.

First, genetic evidence suggests a functional relationship between the *SWI* and *SNF* gene products and the CTD. Strains containing mutations in *SWI* genes exhibit a large number of defects similar to those due to a truncation of the RNA polymerase II CTD (Nonet et al., 1987; Peterson and Herskowitz, 1992; Peterson et al., 1991). In addition, the CTD and the *SWI/SNF* gene products show similar genetic interactions with mutations in *SIN1* and *SIN2*, genes that encode chromatin-associated proteins (Peterson and Herskowitz, 1992; Peterson et al., 1991). Second, the *SRB* gene products have functional and physical interactions with the RNA polymerase II CTD (Koleske and Young, 1995), which has been implicated in the response to activators in yeast and mammalian cells (Allison and Ingles, 1989; Gerber et al., 1995; Scafe et al., 1990). Third, the holoenzyme appears to be responsible for initiating transcription of most, if not all, class II genes in yeast, and the *SWI* and *SNF* gene products are required for transcriptional induction of a large number of genes in vivo (Thompson and Young, 1995; Peterson et al., 1991). Finally, there are perhaps a dozen polypeptides in purified yeast RNA polymerase holoenzyme that have yet to be identified.

We report here that the yeast RNA polymerase II holoenzyme contains SWI2/SNF2, SWI3, SNF5, and SNF11. The SWI/SNF proteins are components of the SRB complex, also known as the mediator, which is tightly associated with the RNA polymerase II CTD. Both the holoenzyme and the SRB/SWI/SNF complex have ATP-dependent nucleosome disruption activities previously ascribed to the SWI/SNF complex. In addition, the holoenzyme facilitates the binding of TATA box–binding protein (TBP) to nucleosomal DNA in an ATP-enhanced manner.

Results

Anti-SRB and Anti-SWI Antibodies Coprecipitate Holoenzyme

SRB regulatory proteins are found tightly and exclusively associated with other components of the RNA polymerase II holoenzyme in cell extracts. If SWI and SNF proteins are subunits of the RNA polymerase II holoenzyme, then antibodies against SRB5 should precipitate both the holoenzyme and SWI/SNF proteins from crude extracts. The results in Figure 1 show that this is indeed the case. SWI2/SNF2, SWI3, and SNF5 proteins coprecipitate with holoenzyme obtained through SRB5 immunoprecipitation. The fraction of SWI and SNF proteins immunoprecipitated from the crude extract appears to be the same as that of the SRB proteins. Control proteins introduced into the crude lysate did not coprecipitate, indicating that the immunoprecipitate was specific for the holoenzyme. When the immunoprecipitation experiment was carried out with antibody against SWI3, essentially identical results were obtained (Figure 1). The SWI/SNF and SRB proteins were immunoprecipitated from the crude extract with similar efficiency whether the immunoprecipitating antibody used was directed against SRB5 or SWI3. A control experiment with antibody against TGFβ failed to precipitate SWI/SNF or SRB proteins. These results indicate that

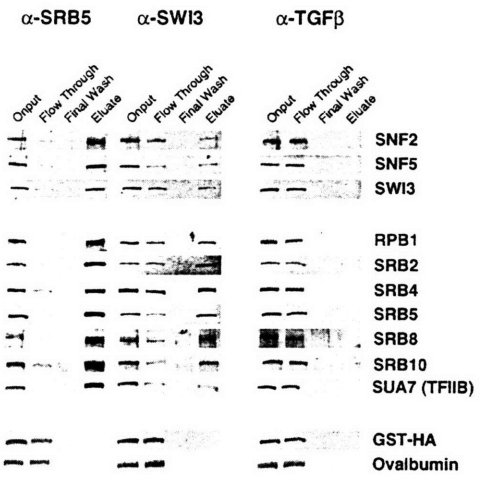

Figure 1. Immunoprecipitation of RNA Polymerase II Holoenzyme from Crude Extracts Using Anti-SRB5 and Anti-SWI3 Antibodies

Immunoprecipitations were from a crude DEAE fraction prepared as described by Hengartner et al. (1995). Immunoprecipitations were carried out with affinity-purified antibodies against SWI3, SRB5, or TGFβ. Ovalbumin and HA-tagged GST were added to each reaction prior to precipitation to serve as controls for specific immunoprecipitation; 1/50 of the onput and flowthrough, and 1/5 of the final wash and eluate were subjected to SDS–PAGE and analyzed by Western blotting using specific antibodies.

SRB and SWI/SNF proteins are tightly associated with one another.

Purified Holoenzyme Contains SWI/SNF Proteins

The immunoprecipitation results led us to investigate whether SWI and SNF proteins are components of purified yeast RNA polymerase II holoenzyme. Antibodies against selected SWI and SNF proteins were used to determine whether these proteins coelute with the RNA polymerase II holoenzyme in the final purification step of the holoenzyme. The data in Figure 2A demonstrate that SNF2/SWI2, SNF5, SWI3, and SNF11 proteins coelute with other known components of the holoenzyme and with transcription activity.

The holoenzyme contains stoichiometric amounts of RNA polymerase II, SRB proteins, and general transcription factors. To ascertain whether the SWI/SNF proteins are stoichiometric components of the holoenzyme, the amounts of SNF2 and SNF5 were estimated by Western blot analysis with various amounts of recombinant proteins as standards (Figure 2B). These data indicate that the purified RNA polymerase II holoenzyme contains approximately equimolar amounts of SNF2, SNF5, and SRB5, the latter being a standard against which other holoenzyme components have previously been compared (Koleske and Young, 1994). Since yeast cells contain between 2000 and 4000 molecules of RNA polymerase II holoenzyme, it appears that there are at least this number of SWI2/SNF2 and SNF5 molecules per cell.

SWI/SNF Proteins Are Components of CTD-Binding SRB Complexes

Genetic evidence indicates that the SRB regulatory proteins and the RNA polymerase II CTD have related functions in transcription initiation and that these involve

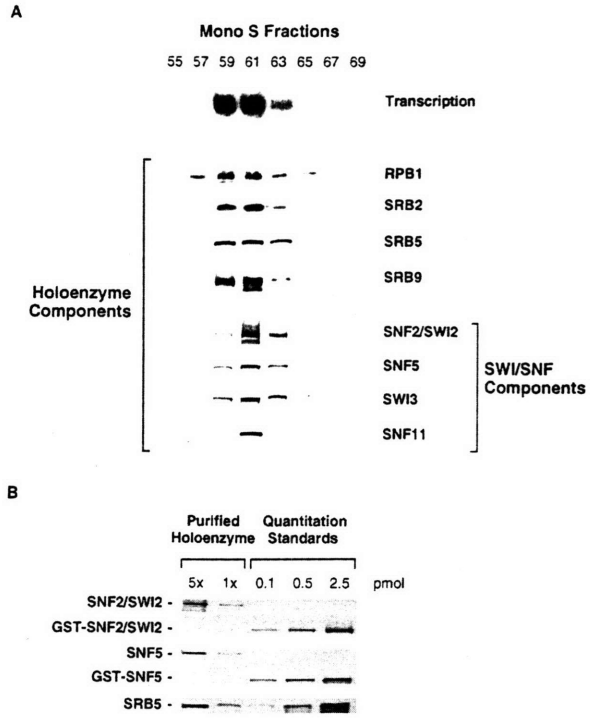

Figure 2. SWI/SNF Proteins Are Components of Purified RNA Polymerase II Holoenzyme

(A) RNA polymerase II holoenzyme eluted from a Mono S column, the last chromatographic step in the purification procedure (Koleske and Young, 1994), was analyzed for transcriptional activity and for the presence of SRB and SWI/SNF proteins by Western blotting.

(B) Quantitative Western blots were used to determine the relative amounts of SRB5 and SWI/SNF proteins in the holoenzyme. Known amounts of recombinant GST–SNF2/SWI2$_{1256-1703}$, GST–SNF5$_{1-193}$, and SRB5 were subject to SDS–PAGE and Western blot analysis along with 2.5 μl and 0.5 μl of purified holoenzyme. There are similar levels of SNF2/SWI2, SNF5, and SRB5 in the purified holoenzyme Previous studies have shown that RPB1 and other SRB proteins are equimolar in purified holoenzyme (Koleske and Young, 1994).

the response to transcriptional regulators (Allison and Ingles, 1989; Gerber et al., 1995; Scafe et al., 1990; Koleske and Young, 1995). Since the SWI and SNF proteins are also involved in activation of a wide variety of genes and since mutations in SWI and SNF genes can produce phenotypes similar to those observed with mutations in SRB genes, we investigated whether SWI and SNF proteins are associated with the SRB complex. The SRB protein complex can be released from the holoenzyme when the latter is treated with monoclonal antibodies against the CTD, and this preparation has been called mediator (Kim et al., 1994). We previously prepared a mediator complex according to the procedure of Kim et al. (1994), confirmed that it has the coactivator activity described by these investigators, and showed that the mediator contains all of the SRB proteins (Hengartner et al., 1995). When this mediator preparation was assayed for the presence of SNF2/SWI2, SNF5, and SWI3 proteins by Western blot, all three SWI/SNF proteins were found (Figure 3).

The SRB complex can also be isolated from crude extracts using a recombinant CTD column (Thompson et al., 1993). An SRB complex was purified extensively by using a recombinant glutathione S-transferase (GST)–CTD column, followed by chromatography with Mono S and Mono Q columns (Figure 4A). The SRB, SWI, and SNF proteins bind to a GST–CTD column, but not to a control GST column, indicating that they bind specifically to the CTD (Figure 4B). Sliver staining and Western blotting confirm that both a multiprotein complex containing SRB proteins and each of the three assayed SWI/SNF proteins coelute from the Mono Q col-

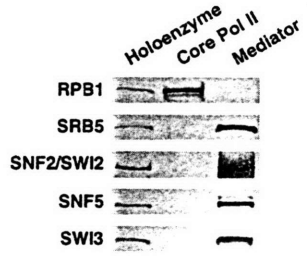

Figure 3. SWI/SNF Proteins Are Present in Mediator Purified Using 8WG16 Monoclonal Antibodies

Mediator was Western blotted along with holoenzyme and core polymerase and probed for the presence of SWI/SNF proteins. The mediator preparation was previously assayed (Hengartner et. al., 1995) and shown to have all transcriptional activities previously described (Kim et. al., 1994).

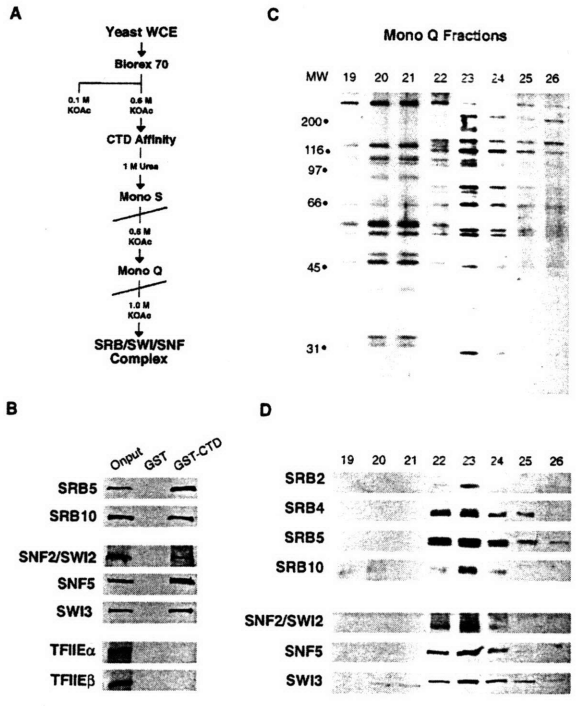

Figure 4. An SRB/SWI/SNF Complex Purified Using CTD Affinity Chromatography

(A) Schematic diagram of the purification.

(B) SRB, SWI, and SNF proteins bind specifically to a GST–CTD column. Western blot analysis of proteins eluted from a GST column and from a GST–CTD column. TFIIE was a negative control for specific retention, as it does not bind GST or GST–CTD.

(C) Silver stain of fractions across the final Mono Q column.

(D) Western blot analysis of SRB and SWI/SNF proteins across the final Mono Q column.

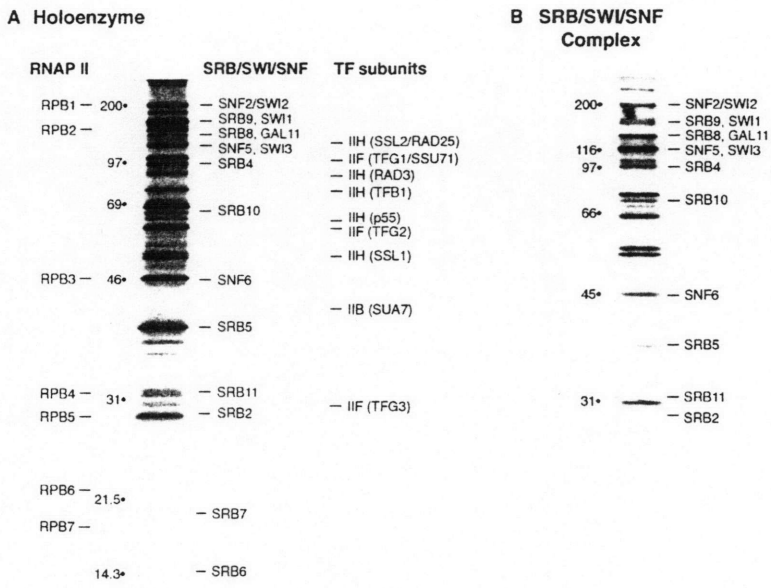

Figure 5. Components of the RNA Polymerase Holoenzyme and the SRB/SWI/SNF Complex

(A) Silver stain of purified RNA polymerase II. Bands that correspond in size to RNA polymerase core subunits, SRB, SWI, and SNF proteins, and general transcription factor IIB, IIF, and IIH subunits are indicated.

(B) Silver stain of the SRB/SWI/SNF complex. Bands that correspond in size to SRB, SWI, and SNF proteins are indicated.

umn (Figures 4C and 4D). There are approximately 25 polypeptides in this complex, and several correspond in size to previously identified SRB, SWI, and SNF proteins (Figure 5). No signals were obtained when Western blots containing the SRB/SWI/SNF complex were probed with antibodies against RNA polymerase II, TBP, TFIIB, or the TFB1 subunit of TFIIH (data not shown). These results indicate that the SRB complex is in fact an SRB/SWI/SNF complex and, furthermore, that the SWI and SNF proteins interact with the holoenzyme, at least in part through their association with RNA polymerase II CTD.

Nucleosome Disruption Activity in Holoenzyme and SRB/SWI/SNF Complex

Previous evidence that SWI1, SWI2, SWI3, SNF5, SNF6, and SNF11 gene products can be isolated as a large multisubunit complex capable of altering nucleosome structure led us to investigate whether the purified RNA polymerase II holoenzyme and the SRB/SWI/SNF complex were able to alter nucleosome structure. Mononucleosome particles were reconstituted from purified histone octamers and a DNA fragment containing two copies of an artificial phasing sequence (Shrader and Crothers, 1989). Digestion of the mononucleosomes with DNase I showed a 10 bp cleavage ladder typical of a rotationally phased nucleosome (Figure 6). Fractions in the last chromatographic step in the purification of the holoenzyme were mixed with mononucleosomes and assayed for the ability to alter nucleosome structure, which can be visualized by changes in the accessibility of the nucleosome to DNase I cleavage. Figure 6A demonstrates that a nucleosome disruption activity coeluted with the RNA polymerase holoenzyme. The ability of the SRB/SWI/SNF complex to alter nucleosome structure was assayed in a similar experiment using fractions from the last step in the SRB/SWI/SNF purification (Figure 6C). The results show that nucleosome disruption activity coeluted with the SRB/SWI/SNF complex. Further analysis of the RNA polymerase II holoenzyme and SRB/SWI/SNF complex showed that the nucleosome disruption activity was ATP dependent (Figures 6B and 6D), as was previously shown for purified SWI/SNF complexes (Côté et al., 1994; Imbalzano et al., 1994; Kwon et al., 1994). In addition, purified core RNA polymerase II showed no nucleosome alteration capability (data not shown). These data indicate that the SRB/SWI/SNF complex contributes chromatin remodeling activity to the RNA polymerase II holoenzyme.

Purified Holoenzyme and the SRB/SWI/SNF Complex Disrupts Plasmid Chromatin

To characterize further the nucleosome disruption capabilities of the holoenzyme and the SRB/SWI/SNF complex, we employed a supercoiling reduction assay (Figure 7). In this assay, chromatin is assembled onto a relaxed closed-circular plasmid that is subsequently purified by glycerol gradient centrifugation. Each assembled nucleosome introduces approximately one negative supercoil to the plasmid, which can be resolved by agarose gel electrophoresis after the removal of histones. When no protein is added to the nucleosome-assembled plasmid, it is highly supercoiled. Fractions from the last column of the holoenzyme purification (see Figure 2A) were tested for their ability to disrupt nucleosome structure and thereby reduce supercoiling in the presence of added topoisomerase I. As can be seen in Figure 7A, this activity coelutes with holoenzyme transcription activity, with the SRB and SWI/SNF proteins (see Figure 2A), and with nucleosome-core disruption activity (see Figure 6A). The supercoiling reduction activity was dependent on ATP (Figure 7A, compare fraction 61 plus and minus ATP), as has been shown for the human SWI/SNF complex (Kwon et al., 1994). Repeating the experiment using fractions from the last column of the SRB/SWI/SNF complex shows that this complex also has an ATP-dependent supercoiling reduction activity (Figure 7B).

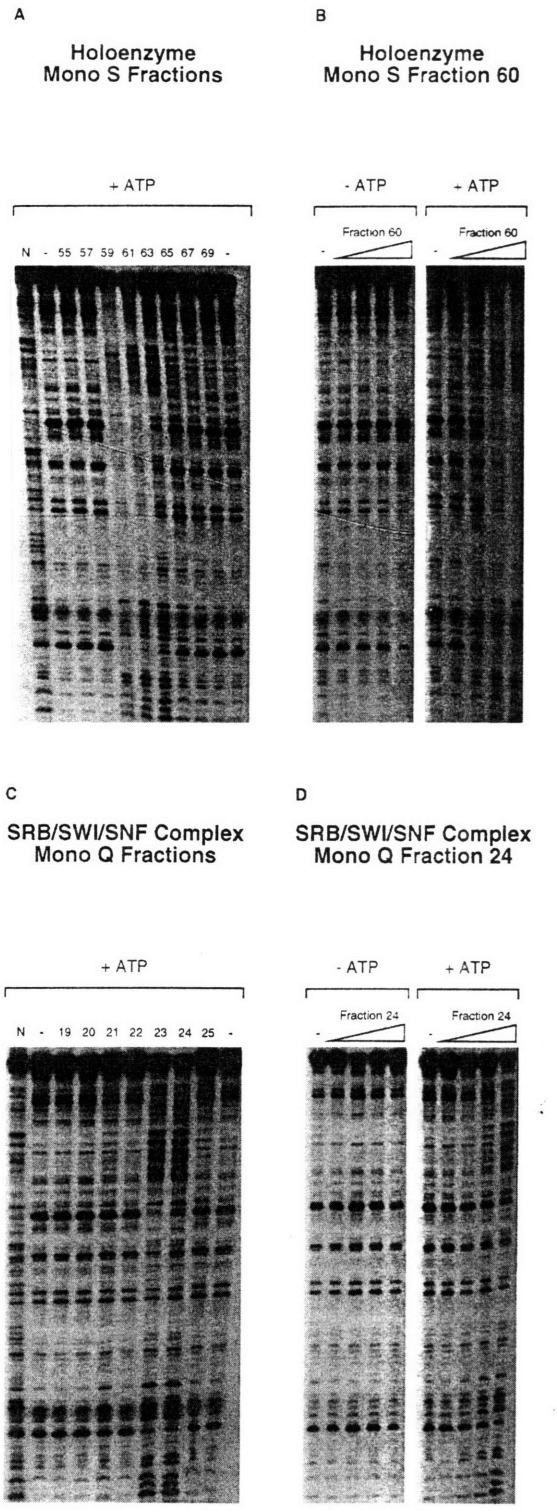

Figure 6. An ATP-Dependent Nucleosomal Disruption Activity Coelutes with the Holoenzyme and the SRB/SWI/SNF Complex

(A) Fractions from the last column of holoenzyme purification (Figure 2A) were assayed for nucleosomal disruption. The peak of nucleosomal disruption activity is in fractions 59–63, coincident with the peak of transcriptional activity.

(B) Purified RNA polymerase II holoenzyme (fraction 60) was titrated for activity with and without 4 mM ATP, as indicated.

(C) Fractions from the final column of the SRB/SWI/SNF complex purification (Figure 4) were assayed for nucleosomal disruption. The peak of nucleosomal disruption activity is in fractions 23 and 24, which is also where the bulk of SRB and SWI/SNF proteins elute.

(D) The SRB/SWI/SNF complex (fraction 24) was titrated for activity with and without 4 mM ATP, as indicated.

Holoenzyme Facilitates the Binding of TBP to Nucleosomes

Previous work has shown that both yeast and human SWI/SNF complexes can facilitate transcription factor binding to nucleosomal DNA containing the relevant factor-binding site (Côté et al, 1994; Imbalzano et al, 1994; Kwon et al, 1994). We tested whether the holoenzyme could increase the binding of TBP to a mononucleosome containing a TBP-binding site. With holoenzyme and ATP present, TBP and TFIIA bound to the mononucleosome at TBP concentrations of 4×10^{-6} M (Figure 8A, lane 7), while no TBP/TFIIA binding was observed in the absence of holoenzyme (Figure 8A, lane 6).

This holoenzyme-facilitated TBP binding might be caused by the stabilizing effects of the additional protein–protein and protein–DNA interactions that occur in the presence of RNA polymerase and general transcription factors, by the ATP dependent nucleosome disruption effects of SWI/SNF, or by a combination of both effects. To address this issue, we tested whether facilitated TBP binding was ATP dependent and observed partial protection of the TATA region on the mononucleosome when ATP is withheld or when ATPγS is used instead of ATP (Figure 8B, lanes 4 and 6). However, addition of ATP enhanced the TBP binding as indicated by the increased protection from DNase I cleavage over the TATA box, the extension of the footprint in the 5′ direction, and the appearance of a hypersensitive band in the 3′ direction (Figure 8B, lane 5). Thus, it appears that the holoenzyme can partially stabilize binding of TBP and TFIIA to a mononucleosome in the absence of ATP. However, the full effect of holoenzyme-facilitated TBP binding requires ATP, presumably because it involves the ATP-dependent nucleosome disruption activity of the SWI/SNF proteins.

Discussion

The RNA polymerase II holoenzyme contains *SWI* and *SNF* gene products, previously identified as global gene coactivators. The SWI and SNF proteins are components of an SRB/SWI/SNF complex, also known as the mediator, which is tightly associated with the RNA polymerase II CTD. Both the holoenzyme and the SRB/SWI/SNF complex have nucleosome disruption activities previously ascribed to the SWI/SNF complex. In addition, the holoenzyme facilitates the binding of TBP to nucleosomal DNA in an ATP-enhanced manner.

Diverse Transcriptional Activators Require SWI/SNF Function In Vivo

Mutations in *SWI1*, *SWI2/SNF2*, *SWI3*, *SNF5*, and *SNF6* cause a substantial reduction in the ability to activate transcription of a wide variety of well-studied genes in yeast cells, including *HO* (Stern et al., 1984), *SUC2* (Neigeborn and Carlson, 1984), *Ty* (Happel et al., 1991),

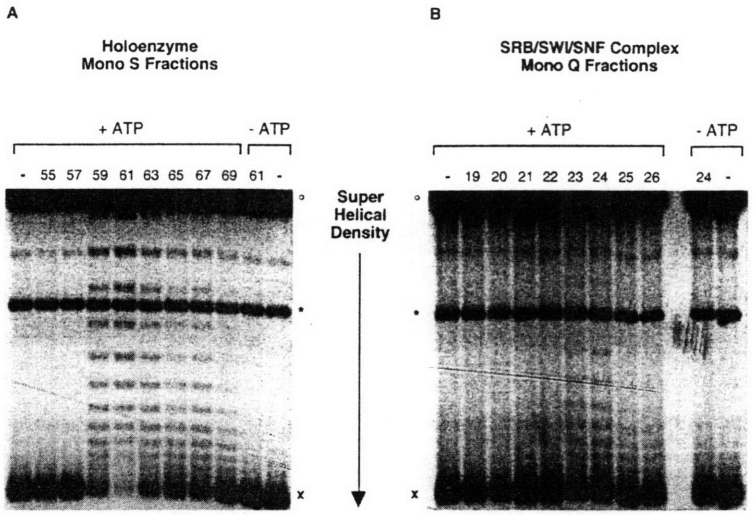

Figure 7. The Holoenzyme and the SRB/SWI/SNF Complex Reduce the Superhelical Density of Chromatin-Assembled Plasmids in an ATP-Dependent Manner

Fractions from the last column of holoenzyme purification (A) and from the last column of SRB/SWI/SNF complex purification (B) were assayed in the presence of 4 mM ATP. Peak fractions of purified holoenzyme and SRB/SWI/SNF complex were assayed with and without 4 mM ATP present as described in Experimental Procedures. The symbols o, *, and x indicate nicked circular plasmid DNA, linear DNA, and highly supercoiled circular DNA, respectively.

INO1 (Peterson et al., 1991), and *ADH1* and *ADH2* (Peterson and Herskowitz, 1992; Taguchi and Young, 1987). For example, *ADH1* and *SUC2* gene expression is reduced by about an order of magnitude in strains in which *SWI1*, *SWI2*, or *SWI3* has been deleted (Peterson and Herskowitz, 1992). Experiments with reporter constructs have revealed that the *SWI* and *SNF* gene products are required for normal responses to a variety of gene-specific activators in yeast such as GAL4, Drosophila *fushi tarazu*, mammalian glucocorticoid and estrogen receptors, and LexA–GAL4 and LexA–Bicoid fusion proteins (Peterson and Herskowitz, 1992; Laurent and Carlson, 1992; Yoshinaga et al., 1992).

We have proposed that the RNA polymerase II holoenzyme is recruited to promoters by activators in vivo (Koleske and Young, 1994). Ptashne and colleagues have shown that recruiting a component of the SRB complex to promoters, presumably in association with the holoenzyme, suffices to obtain activated levels of transcription in vivo (Barberis et al., 1995; M. Ptashne, personal communication). Thus, evidence that LexA fusions with SWI2/SNF2, SNF5, SNF6, and SNF11 proteins are sufficient to activate transcription of a target gene in vivo (Laurent et al., 1990, 1991; Treich et al., 1995) might now be interpreted in terms of holoenzyme recruitment to the target promoter.

We propose that recruitment of the holoenzyme to a specific promoter in vivo provides a means to facilitate TBP binding, regardless of the nucleosome structure at that promoter. The holoenzyme can enhance binding of TBP and TFIIA to a mononucleosome in vitro in the absence of ATP (Figure 8), a result compatible with evidence that the polymerase and general transcription factor components of the holoenzyme provide additional protein–protein and protein–DNA interactions that should stabilize TBP binding (Buratowski, 1994). Holoenzyme-facilitated TBP binding to a mononucleosome is greater in the presence of ATP, which presumably reflects the ATP-dependent nucleosome disruption activity of the SWI/SNF proteins. These observations are consistent with the idea that SWI/SNF protein function is necessary at the subset of promoters whose chromatin structure is particularly restrictive for TBP binding.

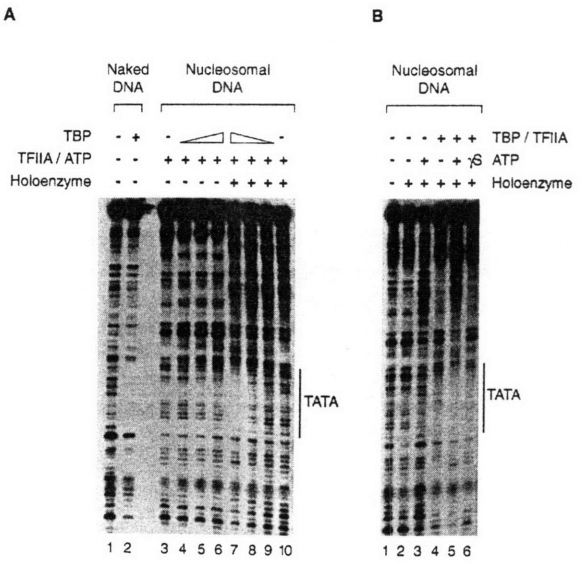

Figure 8. Holoenzyme Facilitates Binding of yTBP and yTFIIA to a Nucleosome Containing a TATA Box in an ATP-Enhanced Manner

(A) Increasing amounts of yTBP in the presence of yTFIIA and 4 mM ATP were tested for the ability to bind to a TATA box containing nucleosome with and without holoenzyme present.

(B) Nucleosomes were incubated with and without holoenzyme, 4 mM ATP or 4 mM ATPγS, yTBP and yTFIIA, as indicated.

SWI/SNF in the Holoenzyme Accounts for Previous Genetic Observations

The presence of SWI/SNF proteins in the RNA polymerase II holoenzyme and the observation that these proteins are components of a subcomplex that interacts with the RNA polymerase II CTD explain several previous observations. SWI/SNF proteins are necessary for transcription activation of many genes in yeast cells (reviewed by Winston and Carlson, 1992; Carlson and Laurent, 1994; Peterson and Tamkun, 1995); CTD truncation adversely affects the response to activators in yeast and mammalian cells (Allison and Ingles, 1989; Scafe et al., 1990; Gerber et al., 1995). Cells with RNA polymerase II CTD truncation mutations, cells with certain *SRB* mutations, and cells with *SWI1*, *SWI2*, or *SWI3*

mutations exhibit remarkably similar phenotypes (Peterson and Herskowitz, 1992; Thompson et al., 1993; Hengartner et al., 1995). The association of the SRB/SWI/SNF complex with the CTD accounts for the observation that cellular defects due to CTD mutations and *SWI* mutations can be alleviated by mutations in *SIN1* and *SIN2*, which encode an HMG1-related protein and histone H3, respectively (Kruger and Herskowitz, 1991; Peterson et al., 1991; Peterson and Herskowitz, 1992).

SRB/SWI/SNF Complex Is Associated with the RNA Polymerase II CTD

The SRB/SWI/SNF complex is tightly associated with the RNA polymerase II CTD. Independent attempts to purify various SRB proteins by column chromatography have always led us to purify the same multiprotein complex: the RNA polymerase II holoenzyme (Koleske and Young, 1994; Koleske et al., 1996; Hengartner et al., 1995; Liao et al., 1995; reviewed by Koleske and Young, 1995). Only very small amounts of SRB protein can be detected that are not associated with the holoenzyme. Two different methods have been described that permit partial purification of an SRB subcomplex. An SRB complex can be isolated using a CTD affinity column (Thompson et al., 1993) or by releasing it from a holoenzyme preparation by using monoclonal anti-CTD antibodies (Kim et al., 1994). Because neither of these preparations is homogeneous, we further purified the SRB complex obtained by CTD affinity chromatography (Figure 4). The SRB and SWI/SNF proteins coelute in the final step of the purification.

We also found that the SRB complex isolated by anti-CTD antibody release contains SWI and SNF proteins. Kim et al. (1994) demonstrated that reconstitution of the response of the holoenzyme to activators required the presence of a subcomplex that could be isolated from holoenzyme with anti-CTD antibodies, which contained SRB2, SRB4, SRB5, and SRB6, and was called the mediator of activation. Our own studies with the mediator, which was purified precisely as described by Kim et al. (1994) and has chromatographic and transcriptional properties identical to those originally described for this subcomplex, revealed that it contained all nine of the known SRB proteins (Hengartner et al., 1995). Thus, the mediator preparation and the SRB complex obtained by CTD affinity chromatography contain very similar, if not identical, complexes.

We have shown that the RNA polymerase II holoenzyme, and its SRB/SWI/SNF subcomplex, contain SWI2/SNF2, SWI3, SNF5, and SNF11. Although we do not have direct biochemical evidence that SWI1 and SNF6 are present in the holoenzyme, other genetic and biochemical data indicate that it is highly likely that SWI1 and SNF6 are also subunits of these complexes (Cairns et al., 1994; Côté et al., 1994; Laurent and Carlson, 1992; Laurent et al., 1991; Peterson and Herskowitz, 1992).

Are There Multiple SWI/SNF Complexes?

Large multisubunit complexes containing yeast SWI and SNF proteins have been purified to varying extents (Cairns et al., 1994; Côté et al., 1994; Peterson et al., 1994). Characterization of two of these preparations by Western blot analysis did not reveal the presence of SRB proteins (Peterson et al., 1994; Cairns et al., 1994). This suggests that the purification procedures employed in these studies separated the SRB and SWI/SNF proteins or that SWI/SNF complexes can exist independent of the holoenzyme.

Since SWI2/SNF2 and SNF5 are stoichiometric components of the holoenzyme and since yeast cells contain 2000–4000 molecules of RNA polymerase II holoenzyme, there are at least 2000 molecules of SWI2/SNF2 and SNF5 molecules per cell. Based on their SWI/SNF complex purification, Côté et al. (1994) estimated that there are between 50 and 150 copies of the SWI/SNF complex in yeast cells. One interpretation of these results is that most SWI/SNF protein resides in the RNA polymerase II holoenzyme, and the form of SWI/SNF complex purified by Côté et al. (1994) is the small amount of SWI/SNF protein that is in the process of assembly into holoenzyme or, alternatively, it represents a subcomplex that can be dissociated from the holoenzyme.

The ability to immunoprecipitate very similar holoenzyme complexes from crude yeast fractions using anti-SRB and anti-SWI antibodies suggests that most of the SWI/SNF protein in these fractions is associated with the holoenzyme. If the SRB and SWI/SNF proteins were in separate complexes, then the relative ratios of SRB and SWI/SNF proteins would differ in the anti-SRB and anti-SWI immunoprecipitates. However, the similar relative ratios of SRB and SWI/SNF proteins found in immunoprecipitates obtained with anti-SRB and anti-SWI antibodies (Figure 1) indicate that the SRB and SWI/SNF proteins are components of the same complex in the crude extract.

SWI/SNF Function Is Highly Conserved in Eukaryotes

SWI/SNF proteins and their functions appear to be highly conserved in eukaryotes. Putative homologs of *SNF2/SWI2* include Drosophila *brahma* and human *hbrm* and *hBRG1*, which have been cloned and implicated in transcriptional regulation (Tamkun et al., 1992; Khavari et al., 1993; Muchardt and Yaniv, 1993). A mammalian homolog of *SNF5*, called *INI1*, has also been cloned (Kalpana et al., 1994). A human SWI/SNF complex has been partially purified that has nucleosome disruption activities similar to those of the yeast SWI/SNF complex (Imbalzano et al., 1994; Kwon et al., 1994). The human SWI/SNF complex contains both hBRG1 and INI1 proteins (Kalpana et al., 1994; G. R. S., unpublished data), as would be expected based on the yeast results. Like the yeast SWI/SNF complex, the human SWI/SNF complex facilitates the binding of activators to nucleosomal DNA.

Implications for Mechanisms Involved in Transcriptional Activation

Our evidence indicates that the RNA polymerase II holoenzyme consists of core RNA polymerase II, all the general transcription factors other than TBP and TFIIA, and a CTD-associated SRB/SWI/SNF subcomplex. The presence of the SRB/SWI/SNF subcomplex in the RNA polymerase II holoenzyme has implications for the

mechanisms involved in transcription activation in vivo. Dynamic competition between chromatin proteins and an activator for a specific DNA site could be resolved in favor of the activator once the SWI/SNF-containing holoenzyme was recruited to the promoter. In this model, the activator and the holoenzyme both contribute to stable transcription initiation complex formation; the activator recruits the holoenzyme by binding to a subset of its components, and the SWI/SNF components of the holoenzyme enhance the stability of the activator–DNA interaction by destabilizing nucleosomes. This model is attractive because it provides a simple solution to the question of how SWI/SNF proteins are brought to promoters and it accounts for the coactivating and nucleosome disruption activities observed in vivo and in vitro for the SWI and SNF proteins.

Experimental Procedures

Immunoprecipitations

All immunoprecipitations were done as described (Hengartner et al., 1995). In brief, 50 μl of the DEAE 400 fraction was diluted 1:4 with modified transcription buffer (MTB) (50 mM HEPES–KOH [pH 7.3], 100 mM potassium acetate, 25 mM MgAc, 5 mM EGTA, 1 μM DTT, 10% glycerol, 0.01% NP-40, 1 mM PMSF, 2 mM benzamidine, 2 μM pepstatin A, 0.6 μM leupeptin, and 2 μg/ml chymostatin) minus the postassium acetate. We added 4 μg of ovalbumin, 4 μg HA-GST, and 2 μg of BSA to each reaction prior to the addition of antibody and then added 0.4 μg of affinity-purified α-SRB5, 0.15 μg of affinity-purified α-SWI3, or 1.5 μg of affinity-purified α-TGFβ to the respective reactions and allowed them to incubate 2 hr at 4°C; 15 μl of goat anti-rabbit covalently linked to magnetic beads (Dynal) were then added and incubated for 1 hr at 4°C with constant agitation. Beads were precipitated with a magnet and washed three times in 200 μl of MTB buffer. The final wash contained no NP-40. Proteins were eluted off the magnetic beads by boiling in 20 μl of sample buffer.

Western Blotting

All Western blots were performed as described (Koleske and Young, 1994). Proteins were detected with the following antibodies: SRB2, SRB4, SRB5, SRB6 (Thompson et al., 1993), SRB8, SRB9 (Hengartner et al., 1995), SRB10, SRB11 (Liao et al., 1995), SWI2/SNF2, SNF5 (gift of B. Laurent), SWI3 (gift of C. Peterson), SNF11 (gift of I. Treich and M. Carlson), TFIIEα, and TFIIEβ (C. J. W. and R. A. Y., unpublished data). Quantitative Western blots were performed as described (Koleske and Young, 1994). Recombinant standards were SRB5 (Thompson and Young, 1995), GST–SNF2/SWI2$_{1256-1703}$, and GST–SNF5$_{1-193}$ (gifts of B. Laurent). GST proteins were purified as described (Smith and Johnson, 1988). Concentrations of recombinant proteins were determined using a colorimetric assay (Bio-Rad) with bovine serum albumin as a standard.

Purification of Holoenzyme and Mediator

Holoenzyme was purified as described (Koleske and Young, 1994). Transcription assays for holoenzyme were done as described (Koleske and Young, 1994). Mediator was purified as described (Hengartner et al., 1995).

SRB/SWI/SNF Complex Purification

Whole-cell extract was prepared from Red Star yeast as described (Thompson et al., 1993). We centrifuged 1.2 liters of the ammonium sulfate pellet for 30 min at 5,000 rpm in an RC3B centrifuge (Sorvall). The pellet was resuspended in 900 ml of buffer A (20 mM K-HEPES [pH 7.6], 1 mM EDTA, 1 mM DTT, 20% glycerol, and protease inhibitors [Thompson et al., 1993]). The suspension was centrifuged again for 30 min at 5,000 rpm in an RC3B centrifuge (Sorvall). The supernatant was mixed with 200 g (dry) of BioRex 70 and stirred for 20 min. The suspension was packed into a column with a 5 cm diameter and washed with 1.5 liters of buffer A plus 100 mM KOAc. Bound proteins were eluted with buffer A plus 600 mM KOAc. Fractions containing protein were pooled, frozen in liquid nitrogen, and stored at −70°C until use. Eluates from two BioRex columns (320 ml, 1.0 g of protein) were thawed and pooled; 320 ml of buffer A plus 2% Triton X-100 were added, and the mixture was centrifuged for 30 min at 12,000 rpm in a GSA rotor (Sorvall). The supernatant was loaded onto a 15 ml CTD affinity column prepared as described (Thompson et al., 1993) at a flow rate of 200 ml/hr. The column was washed with 100 ml of buffer A plus 300 mM KOAc plus 1% Triton X-100, or 100 ml of buffer A plus 300 mM KOAc. Bound proteins were eluted with buffer A plus 300 mM KOAc plus 1 M urea at a flow rate of 25 ml/hr. Fractions containing protein (3.7 mg) were pooled, frozen in liquid nitrogen, and stored at −70°C. The CTD column was equilibrated with buffer A plus 300 mM KOAc plus 1% Triton X-100, and the flowthrough was loaded again. The column was washed and eluted as before. Fractions containing protein (1.8 mg) were pooled, frozen in liquid nitrogen, and stored at −70°C. The CTD eluates were pooled, diluted with 1.5 vol of buffer A plus 0.01% NP-40, and centrifuged for 10 min at 17,000 rpm in an SS-34 rotor (Sorvall). The supernatant was loaded onto a Mono S HR 5/5 (Pharmacia) at a flow rate of 0.3 ml/min. The column was washed with 3 ml of buffer A plus 120 mM KOAc plus 0.01% NP-40. Bound proteins were eluted with a 20 ml gradient of buffer A plus 0.01% NP-40 from 120 mM to 1000 mM KOAc. Fractions were frozen in liquid nitrogen and stored at −70°C until use. Fractions containing SRB4 and SRB5 as assayed by Western blotting were pooled and diluted with 2 vol of buffer B (20 mM Tris OAc [pH 7.6] plus 20% glycerol plus 1 mM DTT plus 0.01% NP-40 plus protease inhibitors). The mixture was centrifuged for 5 min in a microcentrifuge. The supernatant was loaded onto a Mono Q HRR 5/5 column (Pharmacia) at a flow rate of 0.3 ml/min. The column was washed with 1 ml of buffer B plus 200 mM KOAc. Bound proteins were eluted with a 40 ml gradient of buffer B from 200 mM to 2000 mM KOAc. The yield of SRB complex was approximately 100 μg. We analyzed 1 μl of each fraction by silver staining and 7.5–10 μl of each fraction by Western blotting.

Nucleosomal Disruption and Facilitated Transcription Factor Binding Assays

The PH MLT (Figure 6) or PH MLT(+3) (Figure 8) restriction fragments were assembled into rotationally phased mononucleosome particles, purified by glycerol gradient centrifugation, and assayed as described (Imbalzano et al., 1994). At the nucleosome concentrations and reaction conditions employed in this and previous studies, nucleosomes were determined to be stable on the basis of resistance to micrococcal nuclease, the appearance of a 10 bp repeat pattern upon DNase I digestion, and exhibition of reduced mobility upon electrophoresis in native polyacrylamide gels. We have not observed the appearance of free DNA due to nucleosome dissociation in any of our experiments.

In Figure 6, holoenzyme fractions were the same as those used in Figure 1A. In Figure 6A, 0.3 μl of each fraction was assayed in the presence of 4 mM ATP. For the titration of holoenzyme, 0 μl, 0.015 μl, 0.05 μl, 0.15 μl, and 0.5 μl of fraction 60 was used, respectively, with and without 4 mM ATP as indicated. SRB/SWI/SNF fractions were the same as those used in Figures 3C and 3D. In Figure 6C, 1.7 μl of each fraction was assayed in the presence of 4 mM ATP. For the titration in Figure 6D, 0 μl, 0.07 μl, 0.2 μl, 0.7 μl, and 2.0 μl of fraction 24 was used, respectively, with and without 4 mM ATP as indicated.

For Figure 8, binding of yeast TBP (yTBP) and yTFIIA to nucleosomes containing the PH MLT(+3) restriction fragment was performed as previously described (Imbalzano et al., 1994). In Figure 8A, all reactions contained 4 mM ATP. Following a 30 min incubation at 30°C in the presence or absence of holoenzyme (as indicated), increasing amounts of yTBP in the presence of yTFIIA were added. TBP concentrations were 0 (lanes 1, 3, and 10), 0.04 μM (lanes 2, 4, and 9), 0.4 μM (lanes 5 and 8), and 4 μM (lanes 6, 7). yTFIIA (1.5 μM) was also added to all reactions. In Figure 8B, reactions were treated with holoenzyme, alone (lanes 2 and 4), in the presence of 4 mM ATP (lanes 3 and 5), or in the presence of 4 mM ATPγS (lane 6) for 30 min at 30°C, followed by addition of 4 μM yTBP in the presence of 1.5 μM yTFIIA.

Recombinant yTBP was purified as described (Hoey et al., 1990), except that the heparin peak was further purified on a Mono S HR5/5 FPLC column (Pharmacia). Recombinant yTFIIA was purified as described (Ranish et al., 1992).

Supercoiling Reduction Assay

Plasmid chromatin was assembled and purified as described (Kwon et al., 1994). Reactions, total volume 12.5 μl, contained chromatin (2 ng of DNA), 1 U of topoisomerase I (Promega), 2.5 μl of 30% glycerol gradient buffer, 7 μl of buffer A minus KCl, 7 mM $MgCl_2$, 50–100 mM KOAc (final), 4 mM ATP where indicated, and 2 μl of holoenzyme Mono S fractions or 1 μl of SRB/SWI/SNF complex Mono Q fractions. Reactions were stopped after 90 min at 30°C by addition of 6 μl of stop buffer (3% SDS, 100 mM EDTA, 50 mM Tris–HCl [pH 8.0], 25% glycerol, 2 mg/ml proteinase K). Reactions were incubated for 90 min at 37°C and resolved on a 2% agarose gel (50 mM Tris–phosphate [pH 7.3], 1mM EDTA) for 40 hr, at 40 V. Gels were dried and exposed to film.

Acknowledgments

We thank A. Koleske and C. Hengartner for gifts of RNA polymerase II holoenzyme and mediator. We are grateful to C. Peterson for anti-SWI3 and affinity-purified anti-SWI3. We thank B. Laurent for anti-SNF2, anti-SNF5, and recombinant constructs for producing GST–SNF2 and GST–SNF5, and I. Treich and M. Carlson for anti-SNF11. We are grateful to M. Carlson, B. Laurent, C. Peterson, M. Ptashne, P. Sharp, K. Struhl, and F. Winston for stimulating discussions. D. M. C. is a predoctoral fellow of the Howard Hughes Medical Institute. This work was supported by National Institutes of Health grants to R. E. K. and R. A. Y.

Received November 10, 1995; revised December 15, 1995.

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