Association of an activator with an RNA polymerase II holoenzyme

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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 TFIIF (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.

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 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 output (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 Δ 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 immunoprecipi-

tated from a crude yeast protein preparation supplemented with recombinant glutathione S-transferase (GST)–VP16 or GST–VP16 Δ 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 (TGF β) 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 Δ 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-hTGF β -RII (IP 3). Recombinant GST–VP16 was added to the input of IP 1 and IP 3, whereas recombinant GST–VP16 Δ 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^{Δ456FP442} 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^{Δ456FP442} (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^{Δ456}, or GST-VP16^{Δ456FP442}. The columns were washed extensively and GST-fusion and as-

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^{Δ456FP442} glutathione-agarose columns. After washing, the columns were eluted with glutathione. $\frac{1}{200}$ of the load (L) and the flowthrough (FT), and $\frac{1}{10}$ of the last wash (W) and the eluate (E) were subjected to SDS-PAGE and analyzed by Western blot.

A

B

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-VP16^{Δ456}, and GST-VP16^{Δ456FP442} glutathione-agarose columns. After washing, the columns were eluted with glutathione. $\frac{1}{150}$ of the load (L) and the flowthrough (FT), and $\frac{1}{60}$ 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 μ g of RNA polymerase II holoenzyme, and 2.5 and 1 μ g of mediator of activation, respectively.

sociated 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^{Δ456} and no apparent binding to GST-VP16^{Δ456FP442}. 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.

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 TFIID 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 TFIIF, 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 TFIIF (Xiao et al. 1994).

The complexity of the transcription initiation complex suggests that there are numerous molecular mech-

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

Table 3. *Plasmids*

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

in the same gene. Genetic complementation of the recessive alleles involved mating haploids to each other, each containing the CTD truncation mutation *rpb1Δ104* and an independently isolated *srb* mutation, to form diploids and 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 *rpb1Δ104* and a recessive *srb* allele were unable to grow at 12°C and on pyruvate media when transformed with the corresponding wild-type *SRB* allele.

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

DNA methods

DNA manipulations were performed according to Sambrook et al. (1989). Site-directed mutagenesis was performed as described

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

Cloning and sequence analysis

Genomic clones of *SRB7* (pCH2), *SRB8* (pSL301), and *SRB9* (pCH47) were isolated as 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 *rpb1Δ104* and *srb7-1*(Z694), indicating that the correct gene was cloned. Similar results were obtained for the *srb8-1* and *srb9-1* mutant alleles obtained by plasmid gap repair in vivo.

The conditional phenotypes associated with CTD truncations are suppressed both by *srb8-1* or by complete deletion of *SRB8* (Z695 vs. Z701). Similarly, the conditional phenotypes associated with CTD truncations are suppressed both by *srb9-1* or by complete deletion of *SRB9* (Z696 vs. Z703). The mutations in *srb8-1* or *srb9-1* are thus likely to have at least partially destroyed gene function, 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^{Δ456}, and GST–VP16^{Δ456FP442} recombinant proteins were purified from DH5α carrying pGVP, pGVPΔ456, and pGVPΔ456–FP442 (provided by Michael Green,

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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 μ M pepstatin A, 0.6 μ M leupeptin, and 2 μ g/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 μ M pepstatin A, 0.15 μ M leupeptin, and 1 μ g/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 μ M DTT, 10% glycerol, 0.01% NP-40, 1 mM PMSF, 2 mM benzamidine, 2 μ M pepstatin A, 0.6 μ M leupeptin, and 2 μ g/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 1 μ g of recombinant GST–VP16 or GST–VP16 ^{Δ 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 ^{Δ 456}, and GST–VP16 ^{Δ 456FP442} affinity columns were prepared by immobilizing GST fusion proteins on glutathione–agarose beads as described (Smith and Johnson 1988). The columns contained 200 μ l matrix and contained ~300 μ g 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 TGF β -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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Genes Dev. 1995, **9**:

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