

# Temporal Regulation of RNA Polymerase II by Srb10 and Kin28 Cyclin-Dependent Kinases

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## Summary

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

## Introduction

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

The C-terminal domain (CTD) of the large subunit of eukaryotic RNA polymerase II (pol II) contains a repeated heptapeptide that is phosphorylated in a portion of pol

II molecules in the cell (Cadena and Dahmus, 1987; Kodziej et al., 1990; reviewed in Dahmus, 1996). Several lines of evidence indicate that PIC formation involves RNA polymerase II molecules with unphosphorylated CTDs and that these molecules become phosphorylated during or after the transition to active elongation. The form of pol II found in holoenzymes lacks phosphate on its CTD (Kim et al., 1994; Koleske and Young, 1994). The unphosphorylated form of pol II preferentially assembles into a PIC reconstituted with purified transcription factors (Bartholomew et al., 1986; Laybourn and Dahmus, 1990; Lu et al., 1991; Chesnut et al., 1992; Usheva et al., 1992; Kang et al., 1993; Maxon et al., 1994). Since the phosphorylated CTD has a role in recruiting the mRNA capping enzyme to the nascent transcript, and mRNA capping occurs soon after promoter clearance (Coppola et al., 1983; Cho et al., 1997; McCracken et al., 1997a, 1997b; Yue et al., 1997), CTD phosphorylation most likely occurs during the transition from transcription initiation to elongation. Pol II molecules in the midst of elongation contain CTDs that are highly phosphorylated (Bartholomew et al., 1986; Cadena and Dahmus, 1987; Weeks et al., 1993; O'Brien et al., 1994).

The TFIIF kinase is apparently responsible for CTD phosphorylation subsequent to PIC formation (Laybourn and Dahmus, 1990; Ohkuma and Roeder, 1994; Akoulitchev et al., 1995; reviewed in Dahmus, 1996). This view is reinforced by evidence that HIV-1 Tat stimulates transcription elongation by interacting with TFIIF and stimulating CTD phosphorylation (Parada and Roeder, 1996; Garcia-Martinez et al., 1997). Loss-of-function mutations in the yeast TFIIF kinase subunit cause a general defect on class II transcription *in vivo* (Cismowski et al., 1995; Valay et al., 1995), confirming the positive role in transcription inferred from *in vitro* studies.

RNA polymerase II holoenzymes have been purified from yeast and mammalian cells that contain a second CDK implicated in CTD phosphorylation. Genes encoding the Srb10 kinase and its cyclin partner Srb11 were initially identified in a yeast genetic screen designed to reveal factors involved in CTD function; subsequent analysis revealed that their protein products copurify with the other SRB proteins in the RNA polymerase II holoenzyme (Nonet and Young, 1989; Liao et al., 1995). Holoenzymes with a catalytically inactive Srb10 subunit have substantially reduced CTD kinase activity, suggesting that Srb10 is a CTD kinase (Liao et al., 1995), but this has yet to be directly demonstrated. Mammalian Cdk8 and cyclin C are apparently homologs of Srb10 and Srb11, as they share strong sequence similarity and are both found in mammalian holoenzymes (Maldonado et al., 1996; Pan et al., 1997; Scully et al., 1997; D. Chao and R. Y., unpublished data).

While the function of the TFIIF kinase has been thoroughly studied, the function of Srb10 is poorly understood. It is clear, however, that yeast Kin28 and Srb10 CDKs are not functionally redundant. Substantial genetic and biochemical evidence indicates that Kin28 plays an essential, general, and positive role in transcription. In contrast, the evidence suggests that Srb10 is

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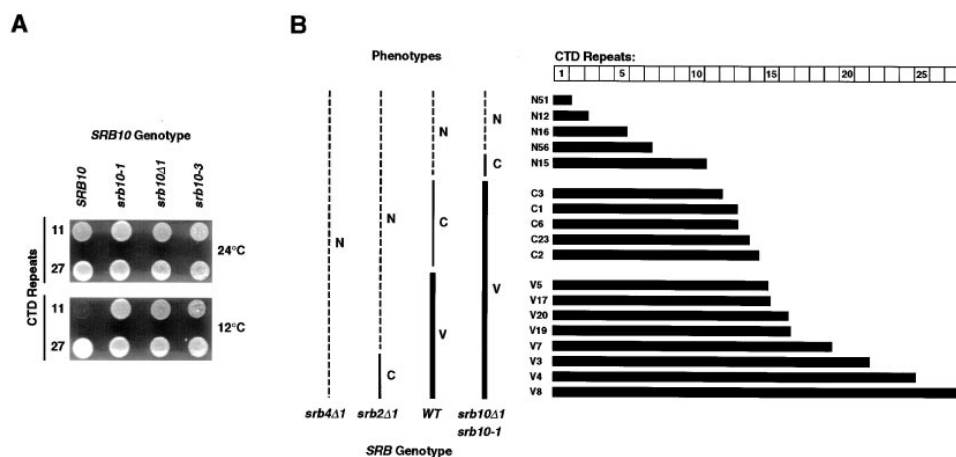


Figure 1. Srb10 Is a Negative Regulator In Vivo

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

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

essential for regulation of a subset of genes that is involved in cell type specificity (Wahi and Johnson, 1995), meiosis (Surosky et al., 1994), and sugar utilization (Kuchin et al., 1995). How Srb10 contributes to the regulation of these important genes is not yet clear.

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

## Results

### Srb10 Is a Negative Regulator of Transcription In Vivo

The *SRB10* gene was identified in a genetic screen designed to reveal genes whose products interact functionally with the RNA polymerase II carboxy-terminal domain (CTD) (Liao et al., 1995). Cells containing a CTD truncation mutation exhibit conditional lethality, and extragenic suppressors (SRBs) were identified that restore the ability of these cells to grow at the nonpermissive

temperature (reviewed in Koleske and Young, 1995). We have found that the suppressing phenotype of the *srb10-1* allele, the original recessive suppressor obtained in the selection, can be duplicated by altering a single amino acid residue in Srb10 that is critical for its kinase function (*srb10-3*; Srb10(D290A)) or by deleting the entire *SRB10* gene (*srb10Δ1*) (Figure 1A). The observation that loss-of-function mutations in *SRB10* can restore viability to yeast cells with a CTD truncation indicates that the Srb10 kinase normally has a negative role in transcription in vivo.

The effect of *SRB10* mutations on yeast cells with a spectrum of CTD truncation mutations (Figure 1B) supports a negative role for Srb10 in transcription. We previously demonstrated that progressive truncation of the RNA polymerase II CTD produced cells with increasingly severe growth phenotypes, and that these phenotypes were due to functional defects rather than reduced stability of the pol II molecules (Nonet et al., 1987; Scafe et al., 1990). The phenotypes exhibited by each of eighteen different strains, which differ only in pol II CTD length, were classified into three categories: nonviable (N) strains that failed to grow under any condition, conditional lethal (C) strains that were cold sensitive, and viable (V) strains that exhibited essentially wild-type growth phenotypes. The wild-type *SRB10* gene was replaced with the *srb10-1* or the *srb10Δ1* allele, and the growth phenotypes of these cells were examined. The results, summarized in Figure 1B, demonstrate that the loss of Srb10 function restores full viability to CTD-mutants that exhibited conditional lethal phenotypes in the presence of wild-type Srb10 kinase activity. In addition, the loss of Srb10 function rescues N15 cells, which were inviable with wild-type Srb10. Thus, the loss of

this putative CTD kinase increases the viability of cells whose pol II molecules have shortened CTDs. In contrast, the loss of *Srb2* or *Srb4*, both positive acting transcription factors, decreases the viability of these cells (Figure 1B). These results provide strong evidence that *Srb10* is a negative regulator of transcription in vivo.

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

#### CTD Phosphorylation by *Srb10* and Kin28 CDKs

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

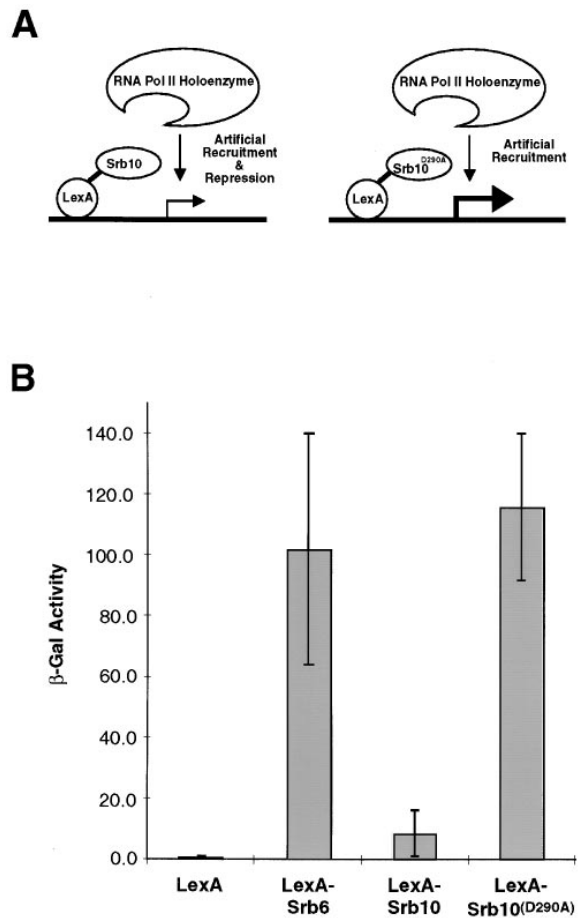


Figure 2. Artificial Recruitment of Holoenzyme with LexA-Srb Fusion Proteins

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

(B) A wild-type strain containing the LexA-lacZ reporter plasmid pSH18-34 was transformed with plasmids expressing LexA alone or LexA fused to *Srb6*, *Srb10*, or *Srb10*(D290A) as indicated. The specific activity of  $\beta$ -galactosidase is expressed in nmoles of *o*-nitrophenol produced per min/mg of total protein assayed. As predicted by the model in (A), the LexA-*Srb10* protein is a very poor artificial activator, whereas the LexA-*Srb10*(D290A) fusion protein is a good activator. The LexA-*Srb10*(D290A) fusion activates as well as LexA-fusions with *Srb* proteins that have positive functions; LexA-*Srb6* is shown as an example. The *Srb10* protein is active in vivo when fused to LexA; the LexA-*Srb10* expression plasmid complements all phenotypes associated with a *srb10* $\Delta$  strain (data not shown). Western blots of whole cell extracts probed with  $\alpha$ -LexA antibodies show that LexA-*Srb10*, LexA-*Srb10*(D290A), and LexA-*Srb6* are all expressed at similar levels.

activity of *Srb10*/*Srb11* and Kin28/*Ccl1* could be directly attributed to the highly purified kinases, since the catalytically inactive CDK mutant kinases were unable to phosphorylate GST-CTD at any level (Figure 4B). These results demonstrate that *Srb10* and Kin28 CDKs are both capable of phosphorylating the CTD.

Genetic evidence presented here and elsewhere (Cismowski et al., 1995; Valay et al., 1995) indicates that

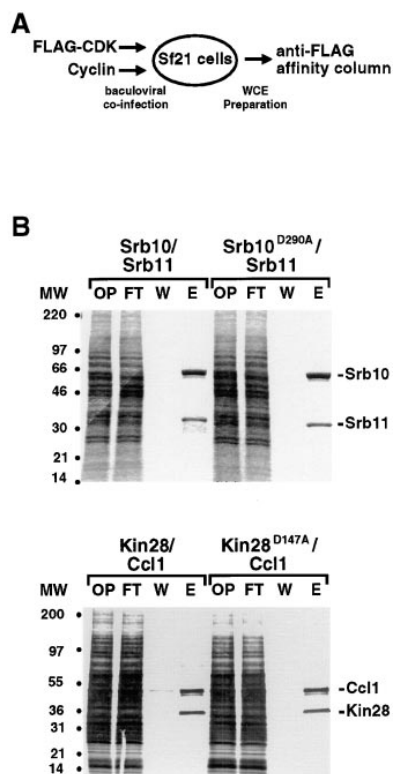


Figure 3. Purification of Recombinant Srb10/Srb11 and Kin28/Ccl1 Cyclin-Dependent Kinases

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

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

Kin28 and Srb10 contribute positive and negative functions, respectively, to transcription *in vivo*. We investigated the possibility that differential phosphorylation of the CTD by the two CDKs might account for their different functions. The amino acid residues of the CTD phosphorylated *in vitro* by the two CDKs were identified by two-dimensional thin layer chromatography. The results demonstrate that both Srb10 and Kin28 phosphorylate serine residues (Figure 4C).

To investigate further the substrate specificity of the two CTD kinases, a battery of synthetic peptides were used as substrates to determine which amino acid residues in the heptapeptide consensus repeat are critical for CTD phosphorylation (Figure 4D). The results show that the activities of Srb10 and Kin28 on these peptide substrates are indistinguishable. Substitution of Ser-2, Thr-4, or Ser-7 with alanine did not significantly affect the ability of the peptide to act as a substrate for either

kinase. In contrast, substitution of Ser-5 with alanine led to a dramatic loss in peptide phosphorylation, suggesting that Ser-5 is the principal phosphoacceptor in the heptapeptide repeat. Substitutions of Tyr-1, Pro-3, or Pro-6 reduced phosphorylation of the synthetic peptides, probably due to the effects such alterations have on their structure. These results indicate that Srb10 and Kin28 CDKs are indistinguishable in substrate specificity and activity in these phosphorylation assays.

### Srb10-Dependent Inhibition of Transcription *In Vitro*

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

This temporal model predicts that holoenzymes with catalytically active Srb10 should be transcriptionally inhibited when the kinase functions prior to association with template DNA. RNA polymerase II holoenzymes containing Kin28 and either wild-type Srb10 or catalytically inactive Srb10(D290A) were purified in parallel and assayed for kinase and transcriptional activities (Figure 5). The two purified holoenzymes contained comparable amounts of Rpb1, Srb2, Srb4, Srb5, Srb10, and Kin28 (Figure 5A). To determine whether Srb10 kinase activity can inhibit transcription by acting prior to PIC formation, we performed an *in vitro* transcription experiment in which both wild-type and mutant Srb10 containing holoenzymes were preincubated with ATP prior to addition of template DNA, additional GTFs, and nucleoside triphosphates (NTPs) (Figure 5B). Preincubation with ATP produced a significant inhibition of transcription with the wild-type holoenzyme but not with the holoenzyme lacking Srb10 catalytic activity (Figure 5B, compare lanes 2 and 4). These data show that transcription by RNA polymerase II holoenzyme is inhibited when the Srb10 kinase is allowed to function prior to PIC formation.

Pol II CTD phosphorylation was monitored in the holoenzymes that were subjected to preincubation with and without ATP (Figure 5B). CTD phosphorylation occurred only in holoenzymes containing catalytically active Srb10 (Figure 5B, compare lanes 2 and 4). Kin28 is apparently not active in the holoenzyme prior to PIC formation, because the Srb10 mutant holoenzyme exhibits essentially no CTD phosphorylation activity during the preincubation. The Srb10-dependent phosphorylation of the CTD was highly efficient; most of the pol II molecules in the wild-type holoenzyme became phosphorylated in

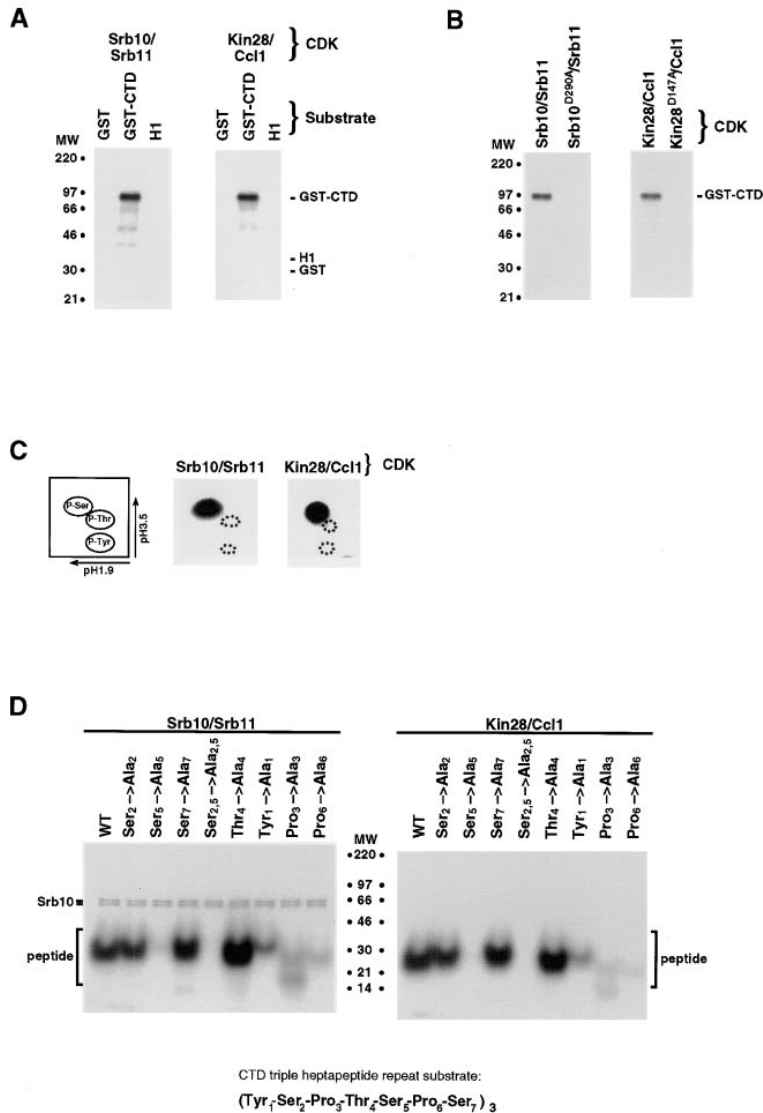


Figure 4. Recombinant CDKs Are Indistinguishable in CTD Phosphorylation Activity

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

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

(C) Phosphoamino acid analysis of in vitro phosphorylated CTD.

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

(D) Holozyme CDKs show identical specificity for synthetic CTD peptide substrates. Synthetic peptides consisting of three heptapeptide consensus repeats, or mutant variations thereof, were used as substrates for recombinant holozyme CDKs. The wild-type (WT) heptapeptide consensus sequence as well as the amino acid numbering used in describing various mutants is shown at the

bottom of the figure. After SDS-PAGE, the phosphorylated peptides were visualized by autoradiography. The Ser-5 to Ala mutant peptide was unable to serve as a substrate for either CDK, strongly suggesting that Ser-5 is the primary substrate labeled by the CDKs.

this reaction (Figure 5B, compare lanes 1 and 2). A control experiment confirmed the specificity of the antibodies used to detect unphosphorylated and phosphorylated CTDs (Figure 5C). Thus, Srb10-dependent CTD phosphorylation is coincident with repression of transcription, a result consistent with previous evidence that formation of a functional preinitiation complex is impaired if the pol II molecules contain phosphorylated CTDs (Lu et al., 1991; Chesnut et al., 1992; Usheva et al., 1992; Kang et al., 1993; Maxon et al., 1994).

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

assayed after the transcription reaction, revealing that CTD phosphorylation occurs in RNA polymerase II molecules from holozymes with or without functional Srb10, albeit the levels are 3-fold less in holozymes lacking catalytically active Srb10 kinase. These results indicate that Srb10-independent CTD phosphorylation occurs during the in vitro transcription reaction, as would be expected from the action of Kin28.

We and others have found that Srb10 is critical for regulation of a subset of genes in yeast cells, including those involved in cell type specificity (Wahi and Johnson, 1995), meiosis (Surosky et al., 1994), and sugar utilization (Kuchin et al., 1995; Liao et al., 1995). Srb10 is not a general repressor of transcription, as a variety of genes are expressed normally in Srb10 mutant cells (Surosky et al., 1994; Liao et al., 1995), and the levels of active holozyme are similar in wild-type and Srb10 mutant cells (S. S. K. and R.Y., unpublished data). The

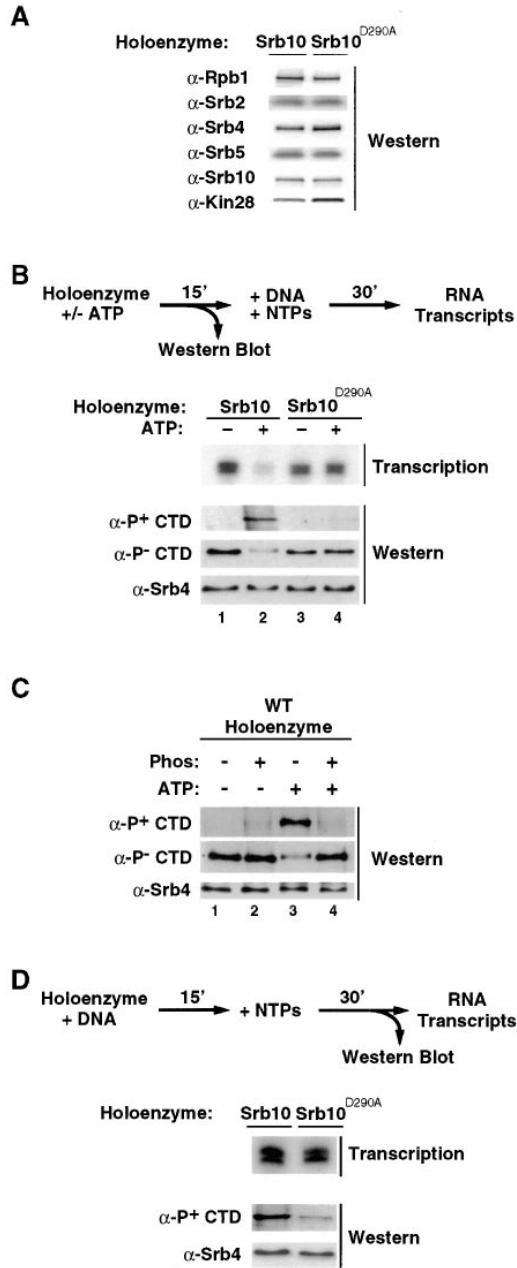


Figure 5. Catalytically Active Srb10 Can Inhibit Transcription by RNA Polymerase II Holoenzyme In Vitro

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

(B) Holoenzymes containing either wild-type Srb10 kinase (lanes 1 and 2) or catalytically inactive Srb10(D290A) kinase (lanes 3 and 4) were preincubated with or without ATP prior to PIC formation and analyzed as diagrammed. Only holoenzymes containing functional Srb10 are inhibited for transcription when kinases are allowed to function before PIC formation (compare lanes 2 and 4). In vitro transcription is performed in the presence of  $\alpha$ -<sup>32</sup>P-UTP resulting in internal labeling of a 400 nucleotide transcript derived from a G-less cassette driven by the *CYC1* promoter. The state of CTD phosphorylation after ATP preincubation was monitored by Western analysis using monoclonal antibodies specific to unphosphorylated ( $\alpha$ -P<sup>-</sup> CTD; 8WG16) or Ser-phosphorylated CTD ( $\alpha$ -P<sup>+</sup> CTD; H5). CTD

observation that Srb10 is not a general repressor of protein-coding genes suggests that in living cells, where there is abundant ATP, Srb10 activity in holoenzymes must be inhibited to prevent constitutive inactivation of the general transcription initiation apparatus. To test this idea, we produced nuclear extracts from wild-type and Srb10(D290A) mutant strains and investigated whether the wild-type extract showed an ATP-dependent inhibition of transcription prior to PIC formation. We previously showed that the transcriptional activity in these extracts is entirely dependent on components of the Srb/mediator complex (Koleske et al., 1992; Thompson et al., 1993), which are tightly associated with pol II holoenzymes (Koleske and Young, 1994). The results, shown in Figure 6, demonstrate that transcription in nuclear extracts is not inhibited by preincubation with ATP, suggesting that these extracts contain an Srb10 inhibitory activity that is lost during holoenzyme purification.

## Discussion

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

phosphorylation occurs during preincubation only in holoenzymes containing functional Srb10 kinase (compare lanes 2 and 4). Control experiments indicate that the Srb10 CTD kinase activity is largely restricted to the holoenzyme in which it resides (data not shown). Srb4 is probed as a loading control.

(C) Changes in MAb reactivity to RPB1 is due to phosphorylation. Holoenzyme containing functional Srb10 was incubated with ATP (lanes 1 and 2). The signal obtained when the phosphorylated preparation is probed with MAb 8WG16 ( $\alpha$ -P<sup>-</sup> CTD) is reduced, and the mobility of RPB1 is retarded (lane 3). The same preparation then reacts with the phospho-serine CTD-specific H5 MAb ( $\alpha$ -P<sup>+</sup> CTD). Subsequent treatment of the sample with protein phosphatase eliminates the H5 reactive band and restores 8WG16 reactivity and mobility to that seen prior to ATP incubation (compare lanes 1 and 4). This indicates the MAbs are accurately probing the phosphorylation state of the CTD.

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

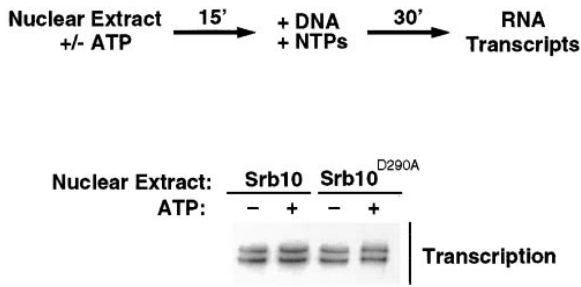


Figure 6. Nuclear Extracts Show No Sr b10-Dependent Inhibition of Transcription by RNA Polymerase II Holoenzyme

Nuclear extracts from cells containing either wild-type Sr b10 kinase or catalytically inactive Sr b10(D290A) kinase were preincubated with or without ATP prior to PIC formation, as diagrammed (the experimental design is identical to that in Figure 5B). In vitro transcription is performed in the presence of  $\alpha$ -<sup>32</sup>P-UTP resulting in internal labeling of a 400 nucleotide transcript derived from a G-less cassette driven by the *CYC1* promoter. No inhibition of transcription was observed after ATP preincubation with either extract.

the CTD, depending on the timing of the phosphorylation event (Figure 7).

In arriving at this temporal model, we first examined the two most obvious models that could account for differential regulation by the two CDKs. It was possible

that the Sr b10 and Kin28 kinases could act on other substrates in the transcription initiation apparatus, but we did not detect phosphorylation of general transcription factors or histones, nor did we find that either kinase could phosphorylate the other. It was also possible that the two kinases phosphorylated different residues on the CTD, but our experiments indicate that they exhibit very similar substrate recognition and modification behaviors. The one clear difference in behavior was the unique ability of Sr b10 to phosphorylate the pol II CTD prior to initiation complex formation when it is a component of the holoenzyme. We conclude that the temporal regulation of transcription by CDKs is an instance where a specific phosphorylation event, carried out at different times, can produce opposite regulatory effects in the cell.

### Negative Regulation by Sr b10 In Vivo

Progressive truncation of the RNA polymerase II CTD produces cells with increasingly severe growth phenotypes (Nonet et al., 1987). The greater the truncation of the CTD, the larger the number of genes affected, accounting for the increasingly severe growth phenotypes (Scafe et al., 1990). The *SRB* genes were originally identified as suppressors of defects due to CTD truncation. A subset of these genes, for example those encoding Sr b4 and Sr b6, is essential for expression of most

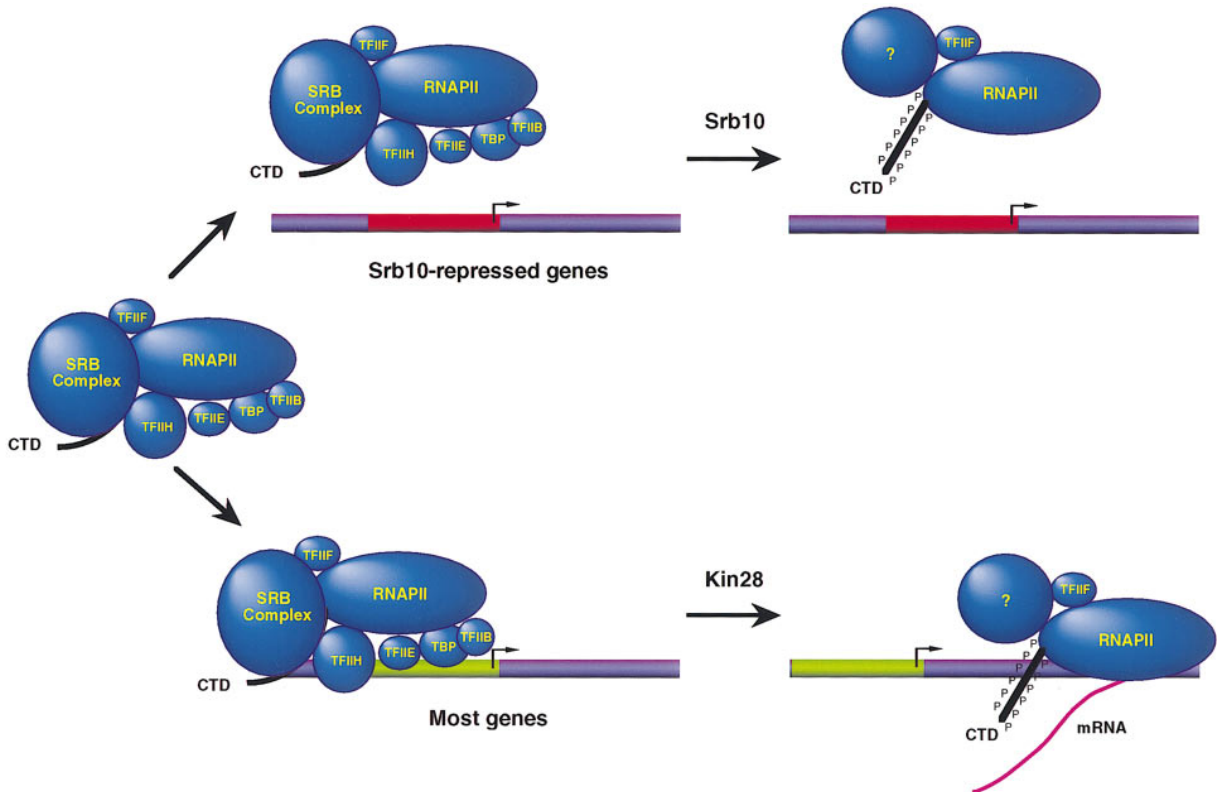


Figure 7. Model for Temporal Function of Holoenzyme CDKs in Transcription Initiation

The two holoenzyme cyclin-dependent kinases are CTD kinases that function at different times. Sr b10-dependent CTD phosphorylation can occur prior to stable preinitiation complex (PIC) formation at a subset of promoters, presumably activated by factors associated with these promoters, with consequent inhibition of transcription. The Kin28 kinase functions after stable PIC formation at promoters generally, producing the hyperphosphorylated form of pol II associated with productive elongation.

protein-coding genes (Thompson and Young, 1995). In contrast, Srb8, Srb9, Srb10, and Srb11 are not essential for expression of protein coding genes generally, but are critical for normal regulation of a subset of genes (Surosky et al., 1994; Kuchin et al., 1995; Liao et al., 1995; Wahi and Johnson, 1995).

Genetic and biochemical evidence indicates that Srb2, Srb4, Srb5, and Srb6 all contribute positively to holoenzyme function (Hengartner et al., 1995; Koleske and Young, 1995). For example, mutations that reduce the function of the Srb2, Srb4, Srb5, Srb6, or Srb7 proteins cause reduced cell viability, and this is exacerbated in cells with CTD truncation mutations (Koleske et al., 1992; Thompson et al., 1993; Hengartner et al., 1995). In contrast, mutations that eliminate Srb10 or Srb11 function actually restore viability to cells with CTD truncation mutations. This, and additional genetic evidence, indicates that Srb10 is a negative regulator of transcription. Highly repressed genes such as *SPO13*, *GAL1*, *SUC2*, *PHO5*, and *MFA2* are derepressed in strains lacking Srb10 activity (Kuchin et al., 1995; Liao et al., 1995; Wahi and Johnson, 1995; S.-M. L. and R. Y., unpublished data). Since Srb10 represses only a subset of genes, there must be a mechanism to activate the kinase solely at these genes. We suggest that promoter-specific factors repress transcription at these genes by stimulating an otherwise quiescent Srb10 prior to stable association of the holoenzyme with promoter DNA (Figure 7).

#### Phosphorylation of CTD by Srb10 and Kin28

Previous studies demonstrated that the kinase activity of purified TFIIF could phosphorylate the CTD (Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992). Previous reports also indicated that Srb10 is involved in CTD phosphorylation, as the *SRB10* gene was identified in genetic selection for suppressors of a CTD truncation defect, and holoenzymes with catalytically inactive Srb10 protein have markedly reduced CTD phosphorylation activity (Liao et al., 1995). The use of highly purified recombinant forms of the two yeast CDKs allowed us to demonstrate that they phosphorylate the CTD, to identify the residues of the heptapeptide that are modified, and to compare and contrast their activities. Srb10/Srb11 and Kin28/Ccl1 phosphorylate the CTD with similar efficiency and are indistinguishable in their specificity towards recombinant full-length CTD or synthetic heptapeptide repeats, down to the specific residue they modify, Ser-5. These results suggest that the positive and negative regulatory functions of the two CDKs are not due to differences in substrate specificity.

#### Temporal Regulation via CTD Phosphorylation

The form of pol II found in RNA polymerase II holoenzyme preparations lacks phosphate on its CTD (Kim et al., 1994; Koleske and Young, 1994). Several experimental observations led us to postulate that the timing of CTD phosphorylation in the holoenzyme determines whether the event has a negative or a positive consequence. The two holoenzyme CDKs have very similar biochemical specificity and activity yet opposite *in vivo* function. In an assay designed to measure transcriptional activity subsequent to template binding, wild-type

and Srb10(D290) mutant holoenzymes are indistinguishable. However, previous studies have shown that the phosphorylation state of the CTD affects PIC formation; formation of such a complex is impaired if the pol II molecules contain phosphorylated CTDs (Lu et al., 1991; Chesnut et al., 1992; Usheva et al., 1992; Kang et al., 1993; Maxon et al., 1994). If one of the holoenzyme kinases phosphorylates the CTD prior to template association, it could inhibit subsequent transcription.

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

#### Regulation of CDKs

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

#### Experimental Procedures

##### Genetic Analysis

To examine the ability of various *SRB10* alleles to suppress the conditional phenotypes caused by a truncated CTD (*rbp1Δ104*), yeast strains Z768 and Z769 (Table 1) were transformed with plasmids containing *SRB10* (pRY2973), *srb10-1* (pRY7091), *srb10Δ1* (pRY2966), and *srb10-3* (pRY7096). Growth conditions were assayed as described (Nonet et al., 1987).

Growth phenotype analysis of yeast cells containing CTD truncation mutations was performed as described (Nonet et al., 1987). The various *SRB10* background strains used were N418 (*SRB10*), Z741



Table 1. Yeast Strains

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

(*srb10Δ1*), and Z735 (*srb10-1*) (Table 1). The viability of cells containing CTD truncations in those backgrounds was assayed by plasmid shuffle, and surviving strains were tested for cold sensitivity.

#### In Vivo Recruitment Assays

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

#### Recombinant CDK/Cyclin Production and Purification from Insect Cells

Recombinant CDK/cyclin pairs were produced using a baculovirus expression system. For expression of CDKs, genes for *Srb10* and *Kin28* were amplified by polymerase chain reactions (PCR) and cloned into baculoviral transfer vectors pSK277 or pSK278 (Koh et al., 1997) to produce recombinants with FLAG epitope-tag at their N termini. For expression of cyclins, genes for *Srb11* and *Ccl1* were amplified by PCR and cloned into baculoviral transfer vectors pBacPAK8 or pBacPAK9 (Clontech). PCRs were performed with Vent DNA polymerase (New England Biolabs). All the PCR clones were verified by DNA sequencing. Mutant CDK clones, *Srb10(D290A)* and *Kin28(D147A)*, were produced by site-directed in vitro mutagenesis (Kunkel et al., 1987) using oligonucleotides CAAAACCTAAGCACC AATTTT and CCTTGCTAGACCGAAAGCTGCTACTTTTATCTG, respectively. All mutations were verified by DNA sequencing.

Recombinant baculoviruses were generated from the recombinant transfer plasmids containing CDKs or cyclins by cotransfection of the plasmids with wild-type viral DNA as recommended by the manufacturer (Clontech). *Spodoptera frugiperda* (*Sf21*) cells were coinfecting with recombinant baculoviruses expressing CDKs and their cyclin partners at a multiplicity of infection (m.o.i.) of 5–10. The cells from 200 mL of culture (approximately  $3 \times 10^9$ ) were collected 60–72 hr postinfection and lysed by sonication. The lysates were then clarified by centrifuging for 3 hr at  $100,000 \times g$  yielding 20–40 mg of total protein in 10 mL. CDK/cyclin pairs were purified from the lysates as described (Koh et al., 1997) using 1 mL of anti-FLAG M2 affinity gel and 73 μg/mL of FLAG peptide in the elution buffer. Typical yields were 0.2%–0.4% of total protein from cell lysate.

#### Kinase Assay

Kinase assays were performed using 1 μg of protein substrate (GST, recombinant GST-CTD, or calf thymus histone H1) or 15 μg of synthetic CTD peptide substrate with 100 ng of pure recombinant CDK/cyclin pairs in 15 μl reaction containing 20 mM Hepes-KOH (pH 7.3), 10% glycerol, 2.5 mM EGTA, 15 mM magnesium acetate, 1 mM DTT, 100 mM potassium acetate, 200 μM ATP, 10 μCi [ $\gamma$ - $^{32}$ P] ATP (NEN, 6000 Ci/mmol, 10 mCi/ml), a mixture of phosphatase inhibitors (1 mM Na<sub>3</sub>N, 1 mM NaF, 0.4 mM NaVO<sub>3</sub>, and 0.4 mM Na<sub>2</sub>VO<sub>4</sub>), a cocktail of protease inhibitors (0.5 mM PMSF and 1 mM benzamide), 1 μM pepstatin, 0.3 μM leupeptin, and 1 μg/ml chymostatin) and 0.5 mg/ml of acetylated BSA. Reactions were assembled on ice and initiated with the addition of ATP. After 60 min at 25°C, the reactions were terminated by adding 15 μl of stop buffer (2× SDS-PAGE loading buffer supplemented with 100 mM Tris-HCl, [pH 6.8],

and 40 mM EDTA) and then resolved by 4%–20% acrylamide gradient SDS-PAGE. The dried gels were exposed directly to autoradiographic films.

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

#### Phosphoamino Acid Analysis

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

#### Nuclear Extract Transcription

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

#### Transcription and Western Blot Analysis

Holoenzyme was purified according to Liao et al. (1995). In vitro transcription reactions were performed essentially as described (Gadbois et al., 1997), with the following modifications. Preincubations (19 μl total) contained all reaction components except TBP, TFIIE, TFIIB, nucleotides, and DNA template; ATP containing reactions were brought to a final ATP concentration of 1 mM with 100 mM stock (Pharmacia). After a 15 min preincubation at room temperature, GTFs (3 μl) and NTP mix (4 μl; 5 mM ATP, CTP, 0.156 mM UTP, 0.25 mM 3'-O-Me GTP and 10 μCi [ $\alpha$ - $^{32}$ P] UTP 3000 Ci/mole) containing 100 ng DNA template (pGALΔ) were added for a final reaction volume of 26 μl. After allowing transcription to proceed for 30 min at room temperature, reactions were stopped by addition of 125 μl stop buffer (10 mM Tris-HCl [pH 7.5], 20 mM EDTA, 2 M ammonium acetate, and 10 μg/ml glycogen) and 150 μl isopropanol. Samples were placed on dry ice for 10 min, microcentrifuged at 14K RPM for 10 min, and pellets resuspended in 6 μl formamide loading dyes and electrophoresed on a 4% Urea-containing denaturing polyacrylamide gel. Gels were dried and exposed to Kodak X-AR film at –80°C with an intensifying screen.

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

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