A kinase–cyclin pair in the RNA polymerase II holoenzyme

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

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

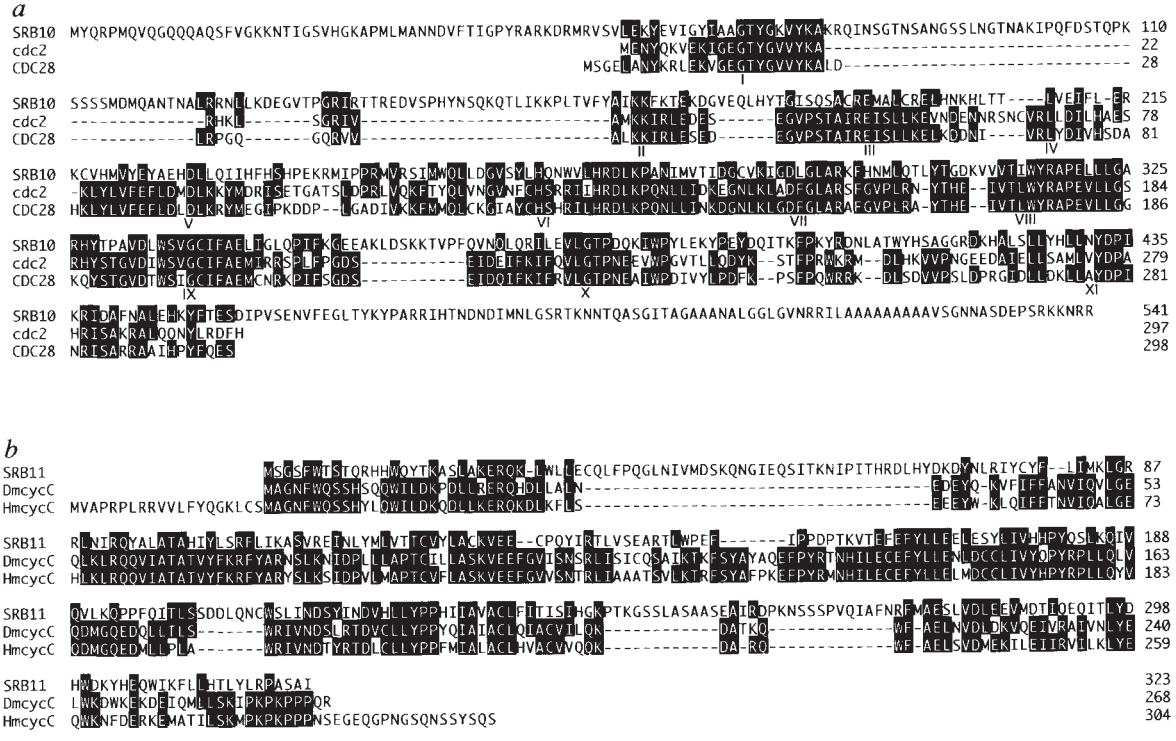

FIG. 1 Sequence of SRB10 and SRB11 proteins and alignment with related kinases and cyclins. *a*, SRB10 sequence alignment with the *S. pombe* Cdc2 and *S. cerevisiae* CDC28 proteins. Identical amino-acid residues are shown as white letters on a black background. Roman numerals indicate the 11 conserved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

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

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

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

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

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

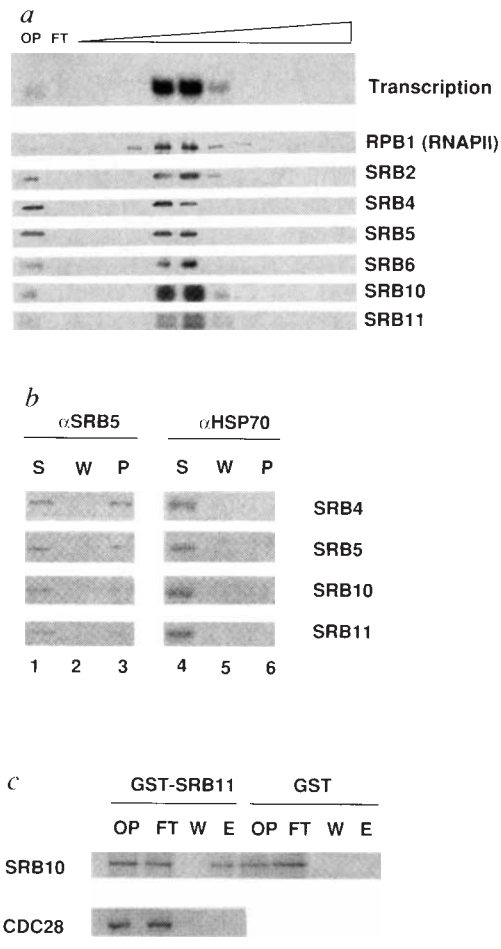

FIG. 2 SRB10 and SRB11 are components of the RNA polymerase II holoenzyme. *a*, RNA polymerase II holoenzyme was purified as described[1]. Holoenzyme loaded onto a Mono-S column, the last chromatographic step in the purification procedure, was eluted with a 0.1–1.0 M gradient of potassium acetate. The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

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

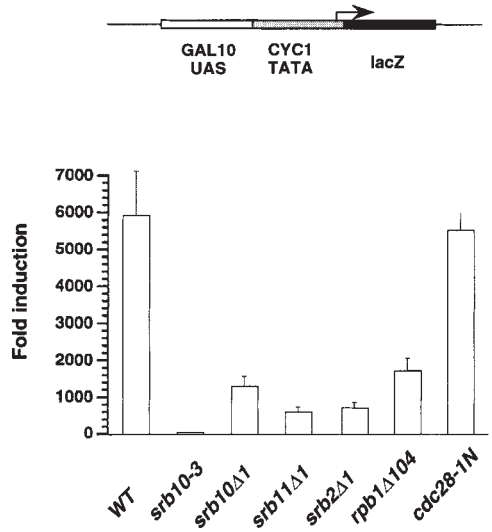

FIG. 3 *SRB10* and *SRB11* mutants are deficient in transcriptional responses at a GAL10 promoter *in vivo*. The relative ability of wild-type cells and *srb10* and *srb11* mutants to respond to galactose induction was measured by expressing *lacZ* under the control of a *GAL10* UAS-containing promoter,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

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

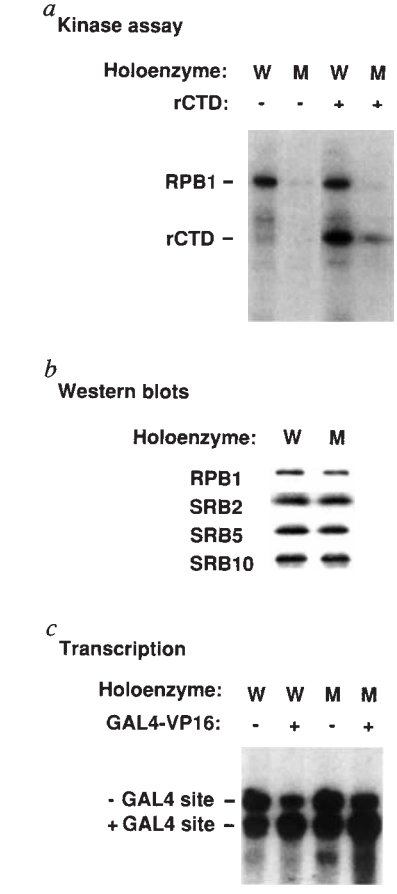

FIG. 4 CTD phosphorylation and transcription *in vitro* with wild-type and mutant RNA polymerase II holoenzymes. *a*, Purified holoenzyme lacking SRB10 function exhibits reduced CTD phosphorylation. Wild-type (W) and mutant (M) holoenzymes were assayed for their ability to phosphorylate the CTD of the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

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

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

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

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

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ERRATA

Dimercaptan–polyaniline composite electrodes for lithium batteries with high energy density

N. Oyama, T. Tatsuma, T. Sato & T. Sotomura

Nature **373,** 598–600 (1995)

FIGURES 3 and 4 were accidentally transposed in this Letter. □

Incorporation of subgenomic amounts of DNA as compensation for mutational load in a gynogenetic fish

Manfred Schartl, Indrajit Nanda, Ingo Schlupp, Brigitta Wilde, Jörg T. Epplen, Michael Schmid & Jakob Parzefall

Nature **373,** 68–71 (1995)

IN Fig. 2*A* of this Letter, the designation of lanes above the gel was incomplete. The correct labelling is shown here. For example, lane 11 contains DNA from *Poecilia mexicana*, which was used as host species to produce the offspring, and not DNA from an offspring, as would have been inferred from the incomplete labelling. □