

The transcriptional coactivator PC4/Sub1 has multiple functions in RNA polymerase II transcription

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Transcription and processing of mRNA precursors are coordinated events that require numerous complex interactions to ensure that they are successfully executed. We described previously an unexpected association between a transcription factor, PC4 (or Sub1 in yeast), and an mRNA polyadenylation factor, CstF-64 (Rna15 in yeast), and provided evidence that this was important for efficient transcription elongation. Here we provide insight into the mechanism by which this occurs. We show that Sub1 and Rna15 are recruited to promoters and present along the length of several yeast genes. Allele-specific genetic interactions between *SUB1* and genes encoding an RNA polymerase II (RNAP II)-specific kinase (*KIN28*) and phosphatase (*FCP1*) suggest that Sub1 influences and/or is sensitive to the phosphorylation status of elongating RNAP II. Remarkably, we find that cells lacking Sub1 display decreased accumulation of Fcp1, altered RNAP II phosphorylation and decreased crosslinking of RNAP II to transcribed genes. Our data provide evidence that Rna15 and Sub1 are present along the length of several genes and that Sub1 facilitates elongation by influencing enzymes that modify RNAP II.

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Introduction

RNA polymerase II (RNAP II) primary transcripts undergo specific and extensive processing, often tightly coordinated with transcription, and which in many cases occurs as transcription proceeds. Additionally, the three major mRNA processing reactions, capping, splicing and polyadenylation, are all linked and can influence each other. Many studies support the idea that RNAP II coordinates these processing steps, and specifically that the carboxy-terminal domain of the RNAP II largest subunit (CTD) is important for targeting of capping enzymes, for enhancing an early step in spliceo-

some assembly and for activating the cleavage/polyadenylation complex at the 3' processing site (reviewed by Hirose and Manley, 2000; Maniatis and Reed, 2002; Proudfoot *et al.*, 2002; Zorio and Bentley, 2004). The CTD thus plays a critical, integrating role in essentially all steps of mRNA synthesis, and in keeping with this is subject to dynamic regulation during the transcription cycle.

The CTD consists of multiple repeats of a seven amino-acid consensus motif, YSPTSPS (Corden *et al.*, 1985; Corden, 1990). This consensus is highly conserved in eukaryotes, although the number of repeats varies among species, from 26 in yeast to 52 in mammals. Five out of these seven residues can be phosphorylated, but serines (ser) 2 and 5 appear to be the major sites of phosphorylation (West and Corden, 1995; Bensaude *et al.*, 1999). RNAP II is hypophosphorylated when recruited to a promoter (designated RNAP IIA), becoming hyperphosphorylated during transcription elongation (RNAP IIO). Experiments involving chromatin immunoprecipitation (ChIP) have provided evidence that differential phosphorylation of ser 2 and 5 coincides with recruitment of factors important for pre-mRNA processing and transcription elongation, and that the pattern of phosphorylation can change, from ser 5 to ser 2, during the course of transcription (Komarnitsky *et al.*, 2000; Schroeder *et al.*, 2000; Cho *et al.*, 2001). Reversible phosphorylation of the CTD thus plays a significant role in modulating the transcription cycle and coordinating the steps of gene expression.

A number of potential kinases and phosphatases have been described that likely target the CTD (reviewed by Kobor and Greenblatt, 2002; Prelich, 2002). Among the kinases, CDK7, CDK8 and CDK9 are well characterized cyclin-dependent kinases that target ser 2 and/or ser 5 residues in the CTD. For example, CDK7 along with cyclin H (Kin28/Ccl1 in yeast) is part of the general transcription factor (GTF) TFIIF. Based initially on studies in yeast, CDK7/Kin28 was shown to phosphorylate the CTD following pre-initiation complex formation, thereby positively regulating early steps in transcription (Feaver *et al.*, 1994; Hengartner *et al.*, 1998). Furthermore, both genetic and ChIP experiments in yeast have provided evidence that Kin28 targets principally ser 5 at the 5' end of genes, facilitating recruitment of capping enzymes and perhaps functioning in coupling transcription and polyadenylation (Komarnitsky *et al.*, 2000; Rodriguez *et al.*, 2000; Schroeder *et al.*, 2000). Several CTD phosphatases have also been described. The best studied of these is Fcp1, which is required for transcription and plays a role in recycling RNAP II for new rounds of transcription (Cho *et al.*, 1999; Kobor *et al.*, 1999). Fcp1 in yeast is chromatin associated both at the promoter and the coding region and has been suggested to function in ser 2 dephosphorylation (Cho *et al.*, 2001). Fcp1 thus appears to play an important role during transcription elongation. More recently, another potential yeast CTD phosphatase, Ssu72, has been identified

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(Krishnamurthy *et al.*, 2004). Ssu72 is an intriguing protein first discovered genetically via interactions with the gene encoding the GTF TFIIB (*SUA7*; Sun and Hampsey, 1996) and subsequently as a component of the mRNA 3' end processing machinery (Dichtl *et al.*, 2002; He *et al.*, 2003; Nedea *et al.*, 2003). Ssu72 may thus play a role in linking transcription with pre-mRNA 3' processing.

Considerable data now exist that transcription by RNAP II is linked to the process of cleavage/polyadenylation, and furthermore that this can involve interactions that initiate at the promoter (reviewed by Calvo and Manley, 2003; Proudfoot, 2004). One protein that has the potential to play a significant role in this process is PC4 (or Sub1 in yeast). PC4/Sub1 (which we refer to here as Sub1) was initially discovered as a transcription factor, biochemically as a coactivator required for activated transcription *in vitro* (Henry *et al.*, 1996) and genetically, analogous to Ssu72, as a suppressor of certain TFIIB mutations (Knaus *et al.*, 1996). Indeed, in light of the fact that both these proteins now seem to be associated with the 3' end machinery, it is noteworthy that the *SUA7* allele specificity of the *SSU72* and *SUB1* interactions is nearly identical (Wu *et al.*, 1999). The suggestion that Sub1 might function in 3' end formation came from the discovery of a physical interaction, conserved from yeast to human, with the polyadenylation factor Rna15 (CstF-64 in humans). The physiological significance of this association was supported by the demonstration of genetic interactions between the two genes showing that deletion or overexpression of *SUB1* suppresses or enhances, respectively, both growth and a transcription termination defect associated with a specific *RNA15* mutant (Calvo and Manley, 2001). These results suggested a model by which Sub1, in association with Rna15, functions as an antitermination factor during transcription. But direct support for this model has been lacking. For example, it remains unclear whether Sub1 and/or Rna15 associate with the elongation complex throughout transcription, nor is it apparent how Sub1 could influence transcription elongation.

We describe here experiments aimed at elucidating more about how Sub1, together with Rna15, might function in transcription elongation. We first provide evidence that both Sub1 and Rna15 are present at the promoter and along the length of several genes. Although not essential, Sub1 enhances, directly or indirectly, recruitment of Rna15 to promoters. Analysis of genetic interactions involving *SUB1* revealed synthetic phenotypes with genes encoding a CTD kinase (*KIN28*) and phosphatase (*FCP1*). Remarkably, this is reflected in a significant decrease in accumulation of Fcp1 in cells lacking Sub1, as well as changes in the phosphorylation status and accumulation of RNAP II. ChIP assays indicate a reduction in both Fcp1 and RNAP II along transcribed genes. These data provide new insights into how Sub1 can function to enhance transcription elongation.

Results

Sub1 and Rna15 are recruited to promoters and remain chromatin associated during transcription elongation

We have proposed a model in which the 3' end processing factor CstF-64/Rna15 interacts with the transcriptional coactivator PC4/Sub1 at promoter regions, and the two then travel together with RNAP II during transcription elongation until

the 3' end processing signals. An important aspect of this model is that Rna15 and Sub1 be localized at active promoters and along the length of transcribed genes. This question can be addressed by ChIP assays, and indeed related experiments have already been performed (Komarnitsky *et al.*, 2000; Licatalosi *et al.*, 2002; Nedea *et al.*, 2003; Kim *et al.*, 2004). However, these studies were not aimed at the specific question we are concerned with, the interactions of Rna15 and Sub1, and in addition reached somewhat contradictory conclusions (see Discussion). For ChIP experiments, yeast strains bearing HA epitope-tagged Rpb3 (an RNAP II subunit), Kin28, Rna15 or Sub1 were utilized. HA-Rna15 was first introduced into *rna15-1* (Bloch *et al.*, 1978; Minvielle-Sebastia *et al.*, 1991) mutant cells, which were grown at 37°C. Full complementation of the slow growth (25°C) and temperature-sensitive (37°C) phenotypes by *HA-RNA15* was observed (data not shown). For others, the appropriate endogenous gene was disrupted by either *LEU* or *URA* and substituted with the HA-tagged version (see Materials and methods; Komarnitsky *et al.*, 2000).

Crosslinked chromatin was isolated, processed, immunoprecipitated and analyzed by PCR. Figure 1A indicates the regions amplified initially from the *ACT1* and *ADH1* genes. HA-Rpb3 and Kin28-HA, which have been analyzed previously (Komarnitsky *et al.*, 2000; Licatalosi *et al.*, 2002), served as controls for the specificity of our assays. As expected, HA-Rpb3 was localized to both the 5' and 3' regions of *ACT1*, while Kin28-HA was predominantly localized to the 5' (promoter) region of the gene (Figure 1B). The inactive silent mating type locus (*HMR1*) or an intergenic region on chromosome V (*V*) served as a negative control, and little or no crosslinking was detected. The pattern of HA-Rna15 and HA-Sub1 crosslinking to *ACT1* was similar to that observed with HA-Rpb3, and significant crosslinking to three regions of *ADH1*, including the promoter, was also detected (Figure 1C, left and center panels, and results not shown). Essentially identical results were obtained when HA-Rna15 was expressed in a wild-type background (Figure 1C, right panel, and results not shown), ruling out the possibility that the mutant Rna15-1 protein may have influenced our results. Given the uncertainty as to whether polyadenylation factors, including Rna15, can be efficiently crosslinked to promoters, we repeated this experiment with primers spanning two additional promoters, from the *PYK1* and *RPS5* genes, and obtained nearly identical results (Figure 1C, right panel). These results indicate that both HA-Sub1 and HA-Rna15 can associate with active genes, both at the promoters and at sites within the transcribed regions.

Given the interactions we have described between Rna15 and Sub1 (Calvo and Manley, 2001), we next investigated whether Sub1 can function in recruitment of Rna15 to active genes. To this end, we introduced a *SUB1* deletion into the HA-Rna15 expressing strain analyzed above, and compared crosslinking of HA-Rna15 to the *ACT1* and *ADH1* genes in the isogenic *SUB1* and $\Delta sub1$ strains (results with *ADH1* not shown). Significant crosslinking was detected at the 5' and 3' ends of both genes in each of the strains (Figure 1D, left), indicating that Sub1 is not essential for HA-Rna15 recruitment. However, quantitation of results from multiple experiments revealed a considerable decrease in crosslinking in the $\Delta sub1$ strain, by approximately 75% at the promoter and 60% in the 3' region (Figure 1D, right). Although RNAP II

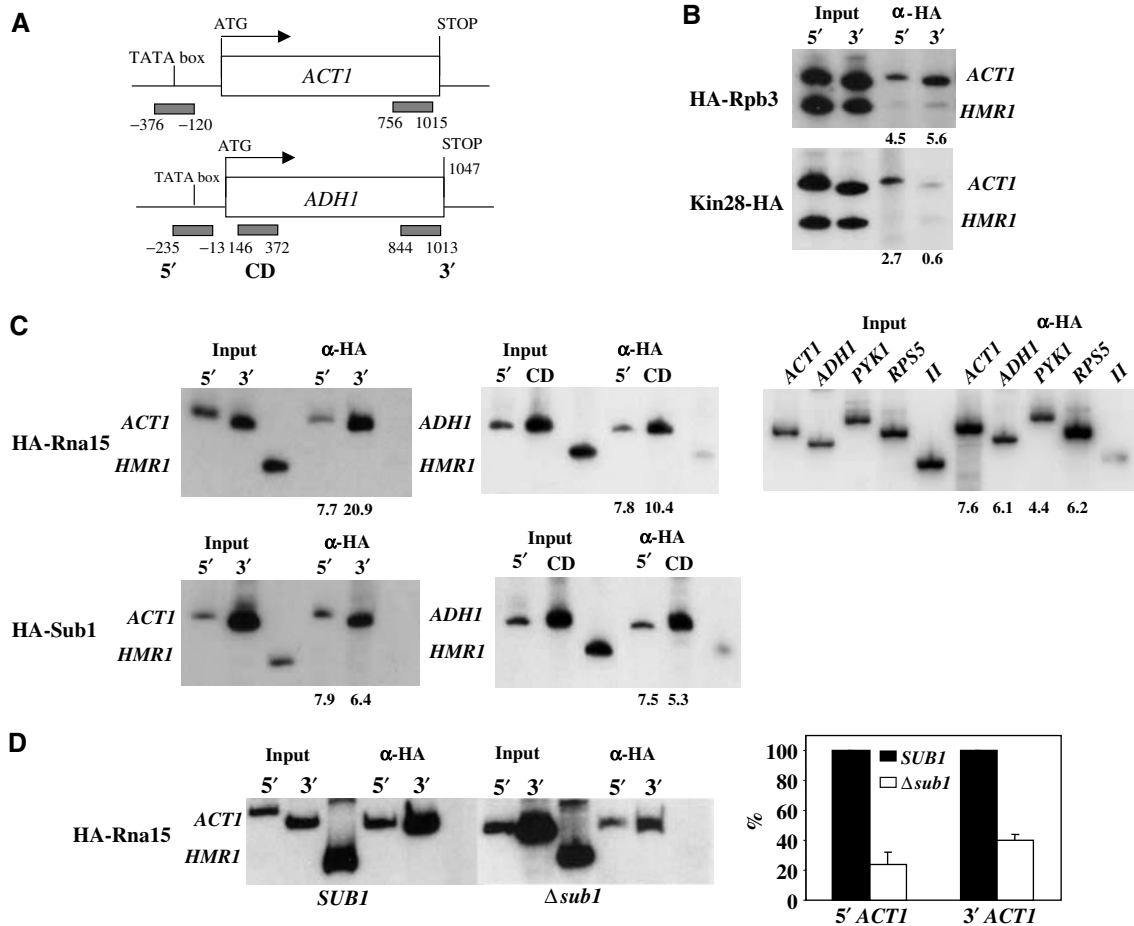


Figure 1 Sub1 and Rna15 are recruited to promoters and remain chromatin associated during transcription elongation. ChIP analysis of HA-tagged Rpb3, Kin28, Rna15 and Sub1 strains to test the presence of these proteins in promoter (5'), coding (CD) and 3' regions of the *ACT1* and *ADH1* genes. (A) Schematic diagram of the *ACT1* and *ADH1* genes: open boxes represent open reading frames and black bars PCR products. (B) ChIP analyses of HA-Rpb3 and HA-Kin28, which serve as positive and negative controls for proteins associated with both 5' and 3' or only with 5' regions, respectively. Primers that amplified nontranscribed regions (*HMR* and *V*) were used as background controls. (C) ChIP analysis of HA-Rna15 and HA-Sub1: the left panel shows Rna15 and Sub1 at 5' and 3' ends of *ACT1*, the middle panel at 5' and coding region of *ADH1* and the right panel 5' regions of *ACT1*, *ADH1*, *PYK1* and *RPS5*, all four in *RNA15* background. Numbers below gels represent adjusted fold over background (see Materials and methods). (D) ChIP analysis of HA-Rna15 from wild-type (*SUB1*) and mutant ($\Delta sub1$) cells. Numbers on the y-axis of the graph represent the percentage of Rna15 localized to 5' and 3' regions of *ACT1* in the $\Delta sub1$ mutant relative to wild-type cells, where Rna15 localization in wild type is considered as 100%.

loading at the promoter was also reduced by *SUB1* deletion (see below), the reduction was relatively modest (~40%) and not sufficient to explain fully the more significant effect on Rna15 recruitment.

The data described above suggest that Sub1 can associate with promoter and transcribed regions of two genes. The promoter association was not unexpected in light of the multiple functions suggested for Sub1/PC4 during transcription initiation, and the association with transcribed regions is consistent with our proposal that Sub1 functions to prevent premature transcription termination (see Introduction). Our results are also in general agreement with ChIP assays performed by Nedeia *et al* (2003), who observed Sub1 association at the promoter and 3' end regions of the *PYK1* gene. However, these authors also observed that Sub1 was present at a reduced level at an internal site in this gene, and therefore suggested that Sub1 might have distinct functions at the 5' and 3' ends of the gene. In light of this, we measured HA-Sub1 distribution by ChIP over the length of a third gene,

PMA1. We utilized six primer pairs, extending from the promoter to just downstream of the poly(A) signal, and the results are shown in Figure 2. Consistent with the results in Figure 1, significant and essentially equivalent Sub1-HA crosslinking was detected across the entire *PMA1* gene. Together, our findings support the notion that Sub1 is associated with the transcription complex throughout elongation.

Sub1 function is connected with CTD phosphorylation

The results we have described here and previously are consistent with the idea that Sub1 associates with the transcription complex and facilitates elongation by RNAP II. While this involves interaction with Rna15, precisely how Sub1 affects elongation is unclear. One possibility is that Sub1 interacts with factors that bind to or modify the CTD. To investigate this idea, we next performed genetic crosses involving *SUB1* and several genes encoding CTD interacting factors.

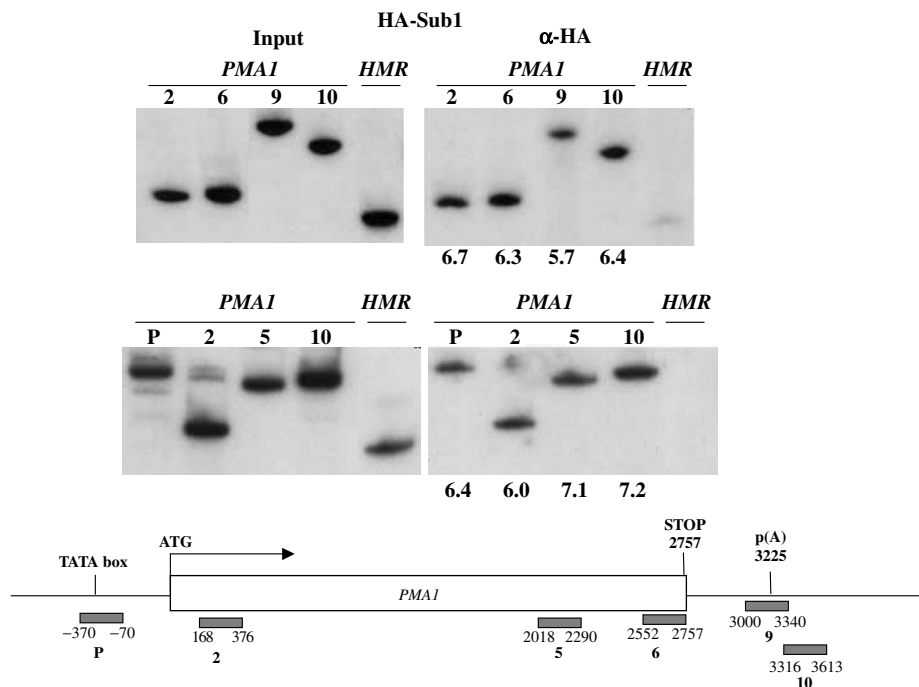


Figure 2 Sub1 is associated with the transcription complex throughout elongation. ChIP analysis of 5', coding and 3' sequences of the *PMA1* gene using an HA-Sub1 expressing strain is shown. Primers that amplified *HMR* were used as background control. The bottom panel shows a schematic diagram of PCR primers used, and the black bars represent PCR products. Numbers below the gels were calculated as described in Figure 1 and Materials and methods.

Kin28 is an essential CTD kinase that functions early in the transcription cycle (e.g., Valay *et al.*, 1995; Komarnitsky *et al.*, 2000; Schroeder *et al.*, 2000) and facilitates recruitment of RNA capping and perhaps polyadenylation factors to the transcription complex (Rodriguez *et al.*, 2000). In light of these properties, we performed a series of genetic crosses with three different *kin28* alleles (*T17D*, *K36A* and *T162A*) and a *SUB1* deletion ($\Delta sub1$). The *kin28-T17D* and *kin28-K36A* mutants display slow growth at 25 and 37°C, and a decrease in kinase activity affecting CTD phosphorylation and capping enzyme recruitment, with *kin28-T17D* giving rise to the strongest phenotypes. The *kin28-T162A* allele, which does not display a slow growth phenotype, decreases kinase activity *in vitro*, but does not detectably affect CTD phosphorylation and capping enzymes recruitment (Espinoza *et al.*, 1998; Kimmelman *et al.*, 1999; Rodriguez *et al.*, 2000). The three double mutants and corresponding parental strains (*kin28* and $\Delta sub1$) were streaked on rich medium plates. The combination of *kin28* mutants with $\Delta sub1$ resulted in either synthetic lethality at 25°C (*T17D*; Figure 3A), reduced growth at 25°C and lethality at 37°C (*K36A*; Figure 3B) or only slightly reduced growth at 37°C (*T162A*; Figure 3C). The synthetic phenotypes of these double mutants thus exactly parallel the decrease in CTD phosphorylation of the single *kin28* mutants. The results establish an allele-specific genetic interaction between *KIN28* and *SUB1*, and importantly show that Sub1 is essential under conditions of decreased CTD phosphorylation.

The above results provide evidence for an interaction between *KIN28* and *SUB1*. Given that a significant aspect of Kin28 activity is to facilitate recruitment and activation of the capping apparatus via the CTD, and that Sub1 interacts with and facilitates recruitment of another RNA processing factor,

Rna15, to the transcription complex, we next tested whether *SUB1* also interacts genetically with a component of the capping apparatus. To this end, we crossed the $\Delta sub1$ strain with a strain bearing a mutation in one of the subunits of the capping complex, the guanylyltransferase, Ceg1. The *ceg1-250* mutation (Fresco and Buratowski, 1996) causes lethality at 37°C, and is localized to the C-terminal domain of Ceg1, which is involved in the interaction with the triphosphatase subunit of the capping enzyme, and with the RNAP II CTD (Cho *et al.*, 1998). Additionally, *ceg1-250* causes lethality when combined with certain *kin28* mutants, including *kin28-T17D* (Rodriguez *et al.*, 2000). The double mutant (*ceg1-250* $\Delta sub1$) and parental strains (*ceg1-250* and $\Delta sub1$) were streaked on rich medium plates. Double mutant cells grew indistinguishably from either single mutant alone (Figure 4). This negative result suggests that the *SUB1-KIN28* interaction is not related to the kinase requirement for capping enzyme recruitment.

The partially overlapping but distinct interactions involving *KIN28*, *CEG1*, *SUB1* and *RNA15* suggested the possibility of an interaction between the genes encoding the capping enzyme, *CEG1*, and the polyadenylation factor, *RNA15*. Such an interaction would provide an intriguing genetic link between the processes of capping and 3' end formation, as well as provide further support for Rna15 function at the promoter region, especially given that Ceg1 is specifically localized at the 5' ends of genes and is released soon after transcription initiates (Schroeder *et al.*, 2000). To investigate this, we crossed the *ma15-1* mutant strain with the *ceg1-250* mutant, and single and double mutants were grown on solid and liquid media at a permissive temperature of 25°C (Figure 4). Interestingly, *ceg1-250*, *ma15-1* cells grew significantly slower than either single mutant, establishing a genetic

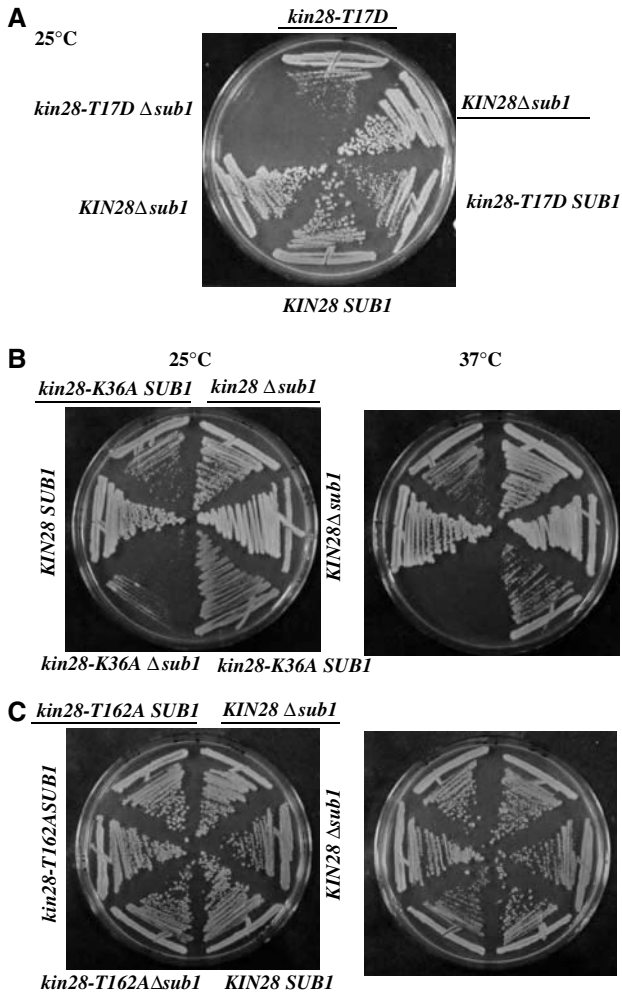


Figure 3 Sub1 function is connected with CTD phosphorylation. We generated *kin28 Δsub1* double mutant strains by crossing a *Δsub1* strain with different *kin28* alleles: (A) *Δsub1* × *kin28-T17D* (YSB592) cross; (B) *Δsub1* × *kin28-K36A* (YSB609) cross; (C) *Δsub1* × *kin28-T162A* (YSB595) cross. Parental strains (*Δsub1*, *kin28-T17D* and *kin28-T162*) and double mutants were streaked on rich medium plates at 25°C (A) or at 25°C (B) and 37°C (C).

interaction between capping and polyadenylation. This result is consistent with our ChIP data showing that Rna15 is present at the 5' regions of transcribed genes.

The above data documenting allele-specific interactions between *KIN28* and *SUB1* provide evidence that Sub1 function is related to phosphorylation of the CTD by Kin28. If this is in fact the case, then *SUB1* might show genetic interactions with genes encoding other factors that affect CTD phosphorylation. One such factor is the CTD phosphatase Fcp1. As we have shown here for Sub1, Fcp1 appears to remain chromatin associated along the length of transcribed genes (Cho *et al*, 2001), and to play a critical role in catalyzing the dynamic changes in CTD phosphorylation that occur during transcription elongation (Cho *et al*, 1999; Kobor *et al*, 1999; Lehman and Dahmus, 2000; Marshall and Dahmus, 2000).

In light of the above, we set out to determine whether we could detect a genetic interaction between *SUB1* and *FCP1*. We generated two *fcp1 Δsub1* double mutant strains by crossing the *Δsub1* strain with two different *fcp1* mutants, *fcp1-1*, with a mutation (R250A, P251A) in a region required for phosphatase activity, and *fcp1-3*, which encodes a protein lacking residues 2–134, which are not required for phosphatase activity (Kobor *et al*, 1999). Figure 5A shows a schematic of Fcp1 (top panel) and the growth phenotypes of the *fcp1* mutants analyzed (bottom panel). The *fcp1-1* mutation caused slow growth at 25°C and lethality at 37°C, while *fcp1-3* mutant cells grew at both temperatures (Figure 5A). We tested the growth phenotypes of the double mutants and corresponding parental strains (*fcp1-1* or *fcp1-3* and *Δsub1*) on rich medium at 25 and/or 37°C (Figure 5B and C, respectively). We also performed growth curves on rich medium for *FCP1*, *fcp1-1* and *fcp1-1Δsub1* strains at 25°C (Figure 5B) and *FCP1*, *fcp1-3* and *fcp1-3 Δsub1* strains at 37°C (data not shown). Deletion of *SUB1* in an *fcp1-1* mutant significantly enhanced the slow growth phenotype at 25°C, but had no effect on the *fcp1-3* mutant at any temperature. These results indicate an allele-specific interaction between *FCP1* and *SUB1* that, as with the *kin28* mutants, correlates with effects on CTD phosphorylation. Together, our findings raise the possibility that Sub1 functions to facilitate RNAP II

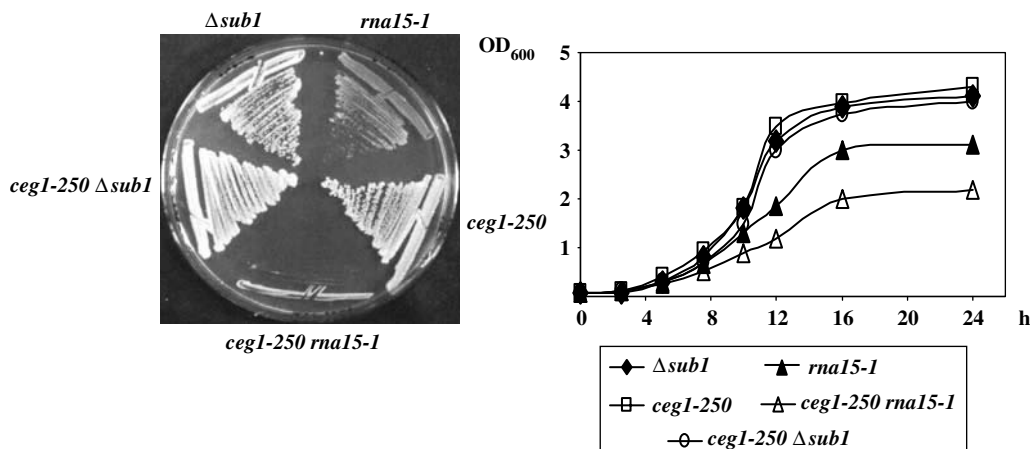


Figure 4 Rna15 and Ceg1 interact genetically. We crossed *ceg1-250* strains (YSB625 or YSB491) with *Δsub1* and *rna15-1* strains. Single and double mutants from both crosses were streaked on rich medium plates at 25°C (left panel) and grown in liquid medium to perform the growth curve (right panel).

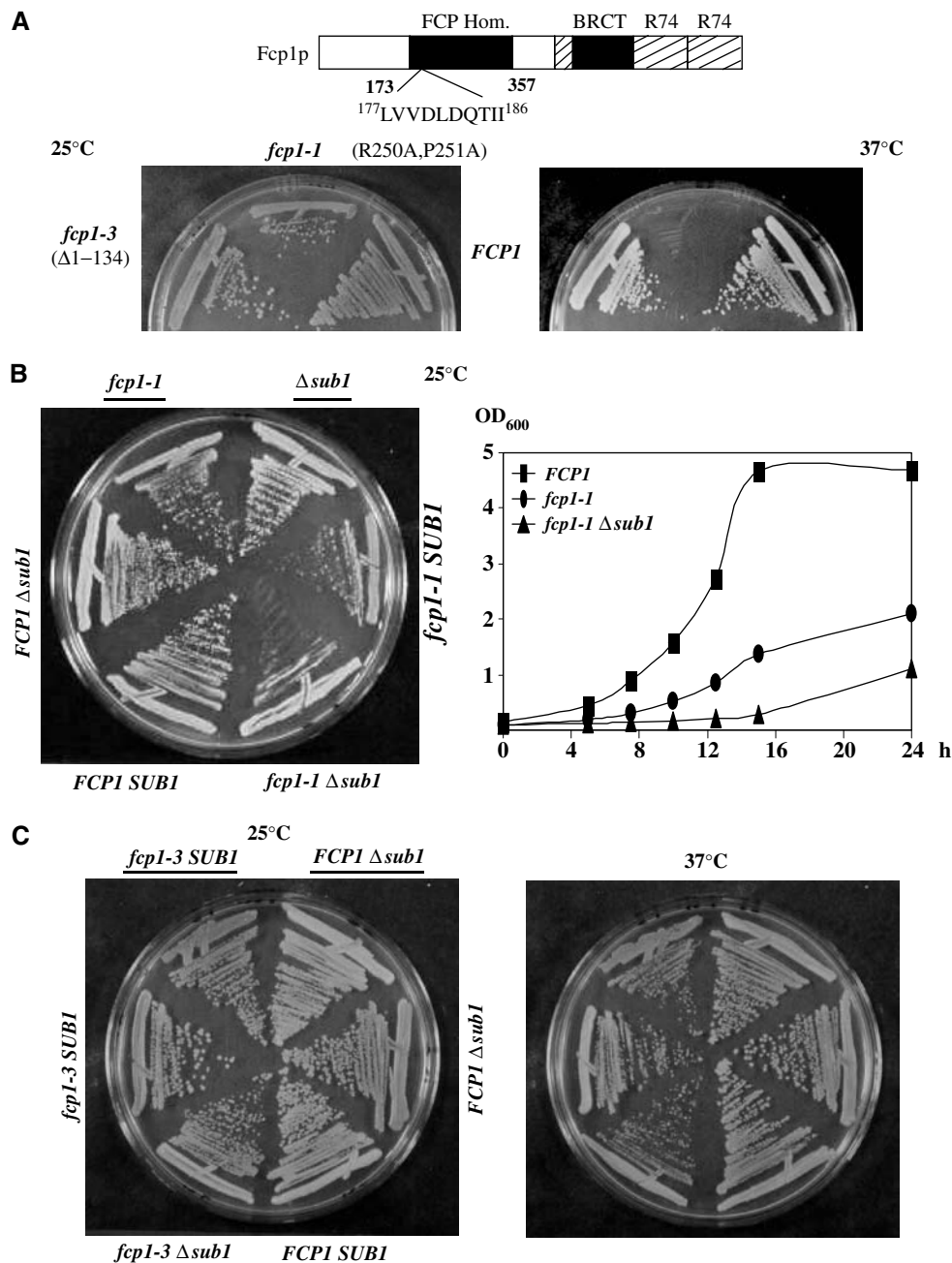


Figure 5 *SUB1* deletion increases the slow growth defect of *fcp1-1* mutant cells. (A) The upper panel is a schematic representation of Fcp1, showing the phosphatase motif (177 LVVDLDQTIH 186) inside the FCP homology region (FCP Hom), the carboxy-terminal domains involved in the interaction with other components of the transcriptional machinery (TFIIB, TFIIF) (represented as R74) and the BRCT domain (Kobor *et al*, 2000). The lower panel shows the growth defects on rich medium for the *fcp1* mutants (*fcp1-1* and *fcp1-3*) at 25 and 37°C; Fcp1 mutated residues are indicated. (B, C) Two *fcp1* Δ *sub1* double mutant strains were generated by crossing a Δ *sub1* strain with either *fcp1-1* strain (YMK20) or *fcp1-3* (YMK28). Growth of double mutants and the parental strains (*fcp1-1* or *fcp1-3* and Δ *sub1*) was tested by streaking them on rich medium at 25°C (B) or at 25 and 37°C (C).

elongation by influencing CTD kinase and/or phosphatase activity.

Sub1 is a regulator of Fcp1 and influences RNAP II accumulation

The above genetic interactions involving *SUB1* and genes encoding Kin28 and Fcp1 suggest that a function of Sub1 may involve modulating CTD phosphorylation. One possibility is that Sub1 helps maintain appropriate levels of one

or more of these proteins. To test this, we prepared lysates from *SUB1* and Δ *sub1* cells and performed Western blots with anti-RNAP II large subunit (LS), -Fcp1 and -HA (to detect Kin28-HA present in this strain) antibodies, as well as control antibodies (Figure 6A). No effect on Kin28-HA levels was detected. RNAP II LS levels were reduced very slightly, but more strikingly, Fcp1 accumulation decreased significantly. This was not due to an effect of *SUB1* deletion on *FCP1* transcription, as Northern blotting revealed

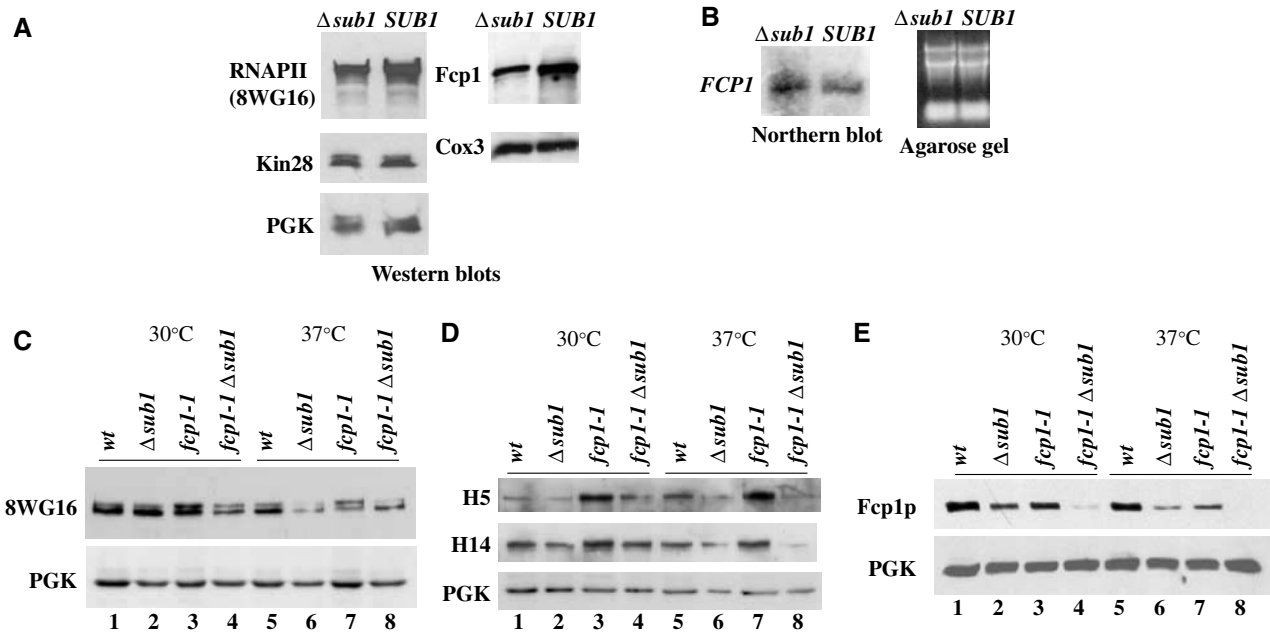


Figure 6 Sub1 is a regulator of Fcp1 and influences RNAP II accumulation. (A) Extracts prepared from isogenic $\Delta sub1$ and wild-type ($SUB1$) cells, YOC07 and YOC06 respectively, were subjected to Western blot and probed with anti-RNAP II, anti-Fcp1 and anti-HA (to detect Kin28p) antibodies. PGK and Cox3 proteins were used as loading controls. (B) Northern blot analysis of total RNAs prepared from $\Delta sub1$ and $SUB1$ cells (left panel), and the corresponding agarose gel showing equal amounts of RNA loaded (right panel). (C, D) Western blots of samples from four spores (same as those used in Figure 5B), grown at 30°C and then for 1 h at 37°C, were probed with three different anti-RNAPII CTD antibodies, 8WG16, H14 and H5 (as indicated). (E) Same samples as in panels C and D, but probed with anti-Fcp1 and anti-PGK (control) antibodies.

equivalent levels of *FCPI* mRNA in the presence and absence of Sub1 (Figure 6B).

To investigate further the effects of Sub1 on RNAP II and Fcp1, we examined in more detail the status of RNAP II, and phosphorylation of the CTD, in wild-type cells and in $\Delta sub1$, *fcp1-1* and $\Delta sub1 fcp1-1$ mutant cells. We utilized for Western blots three antibodies that recognize distinct epitopes in the CTD: 8WG16 recognizes an unphosphorylated epitope and thus detects the Ila isoform, but also recognizes Ilo (Thompson *et al*, 1990; Patturajan *et al*, 1998), while the monoclonals H5 and H4 recognize preferentially phosphorylated ser 2- and ser 5-containing epitopes, respectively (Bregman *et al*, 1995; Patturajan *et al*, 1998; but see Jones *et al*, 2004). At 30°C, *SUB1* deletion alone had little effect on total RNAP II levels or phosphorylation (Figure 6C and D, compare lanes 1 and 2). The *fcp1-1* mutation also had no detectable effect on total RNAP II levels, but resulted in significant increases in H5 and H14 reactivity, suggesting enhanced phosphorylation of both ser 2 and ser 5 residues (Figure 6C and D, compare lanes 1 and 3). Although H5 reactivity (i.e., ser 2 phosphorylation) showed the greatest increase, these results suggest that Fcp1 can dephosphorylate both ser 2 and ser 5 (see Cho *et al*, 2001). The $\Delta sub1 fcp1-1$ double mutant (lane 4) had a striking effect: the amount of the Ila subunit was significantly reduced compared to the wild-type strain as well as both single mutants, while the levels of hyperphosphorylation due to the *fcp1-1* mutation were reduced to wild-type levels. The results at 37°C (Figure 6C and D) were similar, although here $\Delta sub1$ alone revealed a small effect on both Ilo and Ila accumulation (lane 6). *SUB1* deletion entirely suppressed the hyperphosphorylation induced by the *fcp1-1* mutation (lanes 7 and 8). The results

indicate that Sub1 can influence accumulation and phosphorylation of RNAP II.

We also examined levels of Fcp1 in these strains (Figure 6E). *SUB1* deletion reduced accumulation at both 30 and 37°C, somewhat more so than did the *fcp1-1* mutation, previously shown to reduce Fcp1 accumulation by Kobor *et al* (1999) (lanes 1–3 and 5–7). Strikingly, Fcp1 levels were dramatically reduced in the double mutant cells (lanes 4 and 8), becoming undetectable at 37°C. These results indicate that a function of Sub1 is to facilitate accumulation of Fcp1, likely by directly or indirectly increasing its stability. It is noteworthy however that this is not sufficient to explain the effects of *SUB1* deletion on RNAP II phosphorylation (see Discussion).

***SUB1* deletion reduces RNAP II and Fcp1 crosslinking to promoter and coding regions**

The above data provide evidence that Sub1 is required for maintaining optimal levels of Fcp1 and to a lesser extent RNAP II. To determine how this might reflect the presence of these factors on transcribed genes, we carried out ChIP experiments with antibodies against Fcp1 or RNAP II utilizing wild-type and $\Delta sub1$ isogenic strains. Fcp1 crosslinking was significantly reduced in the $\Delta sub1$ strain, as shown here on the *ACT1* and *ADH1* genes, with comparable reductions at both 5' and 3' ends of the gene (Figure 7A). When total RNAP II levels were measured (with 8WG16), crosslinking was also reduced, on both *ACT1* and *ADH1* (Figure 7B). Importantly, reductions were more severe, by roughly threefold, toward the 3' end of each gene, consistent with impaired transcription elongation. However, when phosphorylated RNAP II was measured (with H5), an increase (~2.5-fold) was detected on

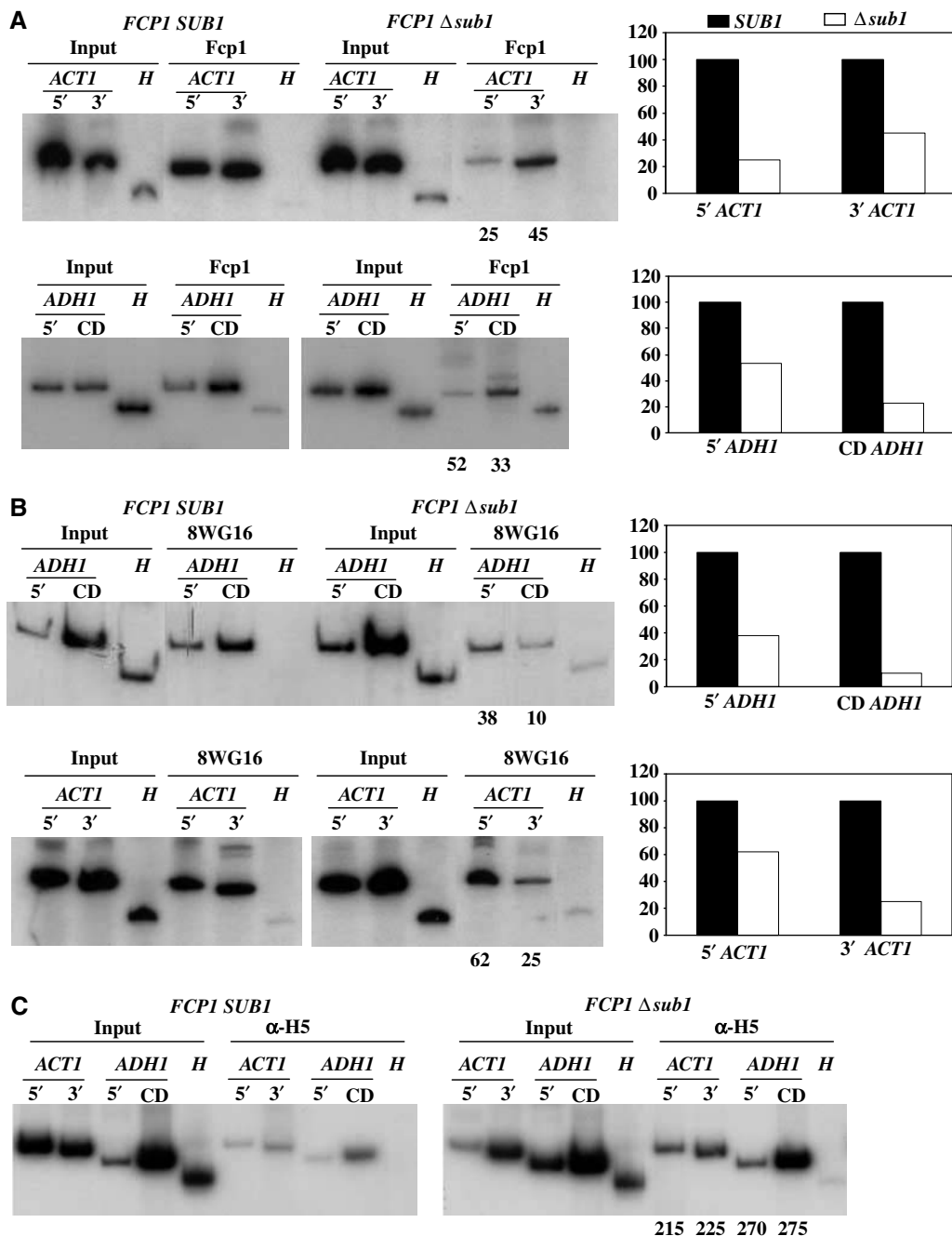


Figure 7 *SUB1* deletion affects RNAP II and Fcp1 crosslinking to promoter and coding regions. ChIP analysis of Fcp1 (A) and RNAP II (B, C) in *SUB1* and $\Delta sub1$ isogenic cells, using anti-Fcp1 (A), 8WG16 (B) or H5 (C) antibodies. PCR primers for *ACT1* and *ADHI* were the same as those used in Figure 1. Numbers under gels and/or graphs compare relative crosslinking of RNAP II or Fcp1 to the 5' and 3' of the *ACT1* gene and 5' and coding region (CD) of the *ADHI* gene in $\Delta sub1$ cells relative to wild-type (*SUB1*) cells, taken as 100.

both genes (Figure 7C), indicating that elongating RNAP II is hyperphosphorylated in the absence of Sub1. Together, these results provide evidence that Sub1 is required for optimal transcription elongation, and that this is due, at least in part, to stabilization of the CTD phosphatase Fcp1.

Discussion

The data we have presented here provide new insights into how factors initially described as participating in disparate

aspects of gene expression can function together in unexpected ways. Specifically, we have provided considerable support for the idea that polyadenylation factors, such as Rna15, can stably associate with chromatin at or near gene promoters and remain associated along the length of transcribed genes. Our data strengthen the case that this is an important, evolutionarily conserved aspect of gene expression. Our findings that the transcriptional coactivator Sub1 is important for this to occur optimally strengthen the significance of the Rna15/Sub1 interaction we described previously.

Our data also provide considerable support for our hypothesis that Sub1 functions to prevent premature transcription termination. The presence of Sub1 along the length of transcribed genes indicates a direct interaction between Sub1 and the elongating RNAP II complex, and the ability of Sub1 to interact with both a CTD kinase and phosphatase suggests a novel function: to regulate CTD modifiers during transcription.

Considerable data have accumulated over the last few years addressing the question of whether polyadenylation factors associate with transcription factors at promoters, and/or with elongating RNAP II. Perhaps the earliest evidence supporting this came from experiments utilizing human cell extracts, which showed that the polyadenylation factor CPSF could be immunopurified in a relatively stable complex with the GTF TFIID, and then in reconstituted transcription reactions was transferred to elongating RNAP II (Dantonel *et al*, 1997). This was consistent with other experiments indicating a direct role for the CTD in 3' end formation (McCracken *et al*, 1997; Hirose and Manley, 1998; Additionally, immunostaining experiments examining lampbrush chromosomes in frog oocytes (Gall *et al*, 1999) and polytene chromosomes in flies (Osheim *et al*, 2002) revealed the presence of polyadenylation factors along the entire length of transcribed genes, coincident with RNAP II. To our knowledge, ChIP experiments to localize 3' end factors in metazoan organisms have not been reported.

Experiments in yeast have also provided support for the presence of polyadenylation factors at the 5' ends of genes, although the evidence has not been conclusive. Intriguingly, immunopurification of yeast TFIID also found CPSF polypeptides associated with it (Sanders *et al*, 2002). However, in these experiments, antibodies directed against multiple TFIID subunits were utilized, and the CPSF subunits were detected with only one. ChIP experiments have produced somewhat conflicting results. Initial experiments using antibodies directed against several different polyadenylation factors failed to detect chromatin crosslinking (Komarnitsky *et al*, 2000). However, Licatalosi *et al* (2002), also using antibodies directed against specific polyadenylation factors (Rna15 was not tested), detected crosslinking at both the 5' and 3' ends of genes, with somewhat stronger signals toward the 3' ends. The authors suggested from this data that polyadenylation factors begin to be recruited at or near promoters and then throughout the length of the transcribed gene. More recently, Kim *et al* (2004) performed a thorough analysis of the association of a number of TAP-tagged polyadenylation factors (including Rna15) along the length of several genes. They observed low to modest levels of crosslinking at promoters and along the lengths of genes until the 3' processing signal was reached, at which point dramatic increases in crosslinking were detected. The authors suggested that while their findings did not exclude recruitment of polyadenylation factors to initiating and/or elongating RNAP II complexes, a simpler explanation was that such factors were recruited only at the 3' ends of nascent transcripts.

Our results provide strong support for a model in which certain polyadenylation factors, for example, Rna15, are in fact recruited to promoters and remain chromatin associated along the length of at least a subset of transcribed genes. By using HA-tagged proteins, we provided evidence not only that Rna15 is present at and downstream of promoters, but also,

as judged by crosslinking efficiency, at levels at least comparable to RNAP II (i.e., Rpb3) itself. In each case, levels significantly above background crosslinking were detected. Notably, these values were only two- to three-fold greater than those observed by Kim *et al* (2004) and differences could be attributed to any number of variations in experimental conditions, for example, use of TAP versus HA epitope tags. What was most striking however was that these values appear small relative to the signals detected at and just downstream of 3' end sites, which were as high as 60- to 70-fold above background (Kim *et al*, 2004; note that our experiments were not designed to detect interactions at the polyadenylation signals). These values are unusually high for ChIP assays and, rather than suggesting that the more typical upstream crosslinking may not be indicative of significant interactions, point to a major change in the polyadenylation complex that allows especially efficient detection by ChIP assays. This could include recruitment of additional factors necessary for 3' processing that stabilize the complex, and/or dissociation of other factors present during elongation that cause the RNA processing factors to become more accessible. These changes likely involve the switch from ser 5 to ser 2 CTD phosphorylation that occurs during elongation. Ser 2 phosphorylation is important for one polyadenylation factor, Pcf11, to bind the CTD (Licatalosi *et al*, 2002; Meinhart and Cramer, 2004), and reductions in ser 2 phosphorylation both decrease 3' end formation in yeast and flies (Ahn *et al*, 2004; Ni *et al*, 2004) and prevent the high-efficiency crosslinking at the 3' processing site (Ahn *et al*, 2004).

Our data have provided evidence that Sub1 is also present across the length of several transcribed genes. Although consistent with our proposal that Sub1 can function as an antitermination factor, they differ from a recent suggestion that Sub1 may be present only at the 5' and 3' ends of genes (Nedeia *et al*, 2003). This conclusion was based on an observed decreased ChIP signal at a single site within the *PYK1* gene, and led to the suggestion that Sub1 has distinct functions at the 5' and 3' ends of genes (Nedeia *et al*, 2003; Kim *et al*, 2004). This drop off in crosslinking could reflect a specific property of the *PYK1* gene or the primers utilized in PCR. Nonetheless, it is in fact likely that Sub1/PC4 has multiple functions in gene expression (and perhaps other nuclear processes; see Wang *et al*, 2004), including its well-studied transcriptional coactivator function. Whether or not Sub1 has a distinct function at the 3' ends of genes remains to be determined.

How does Sub1 function to enhance transcription elongation? The genetic and biochemical data presented here provide evidence that this involves interaction with CTD modifiers such as Kin28 and Fcp1. Most striking was the significant decrease in Fcp1 accumulation brought about by *SUB1* deletion. This resulted in enhanced phosphorylation of RNAP II on two genes, which likely contributed to the observed elongation defects. But the fact that total RNAP II levels were not significantly enhanced suggests a complex role for Sub1 in influencing CTD modification, and we propose a model whereby Sub1 influences both CTD kinase and phosphatase activity. Our experiments showed that the *fcp1-1* mutation enhanced CTD phosphorylation, a not unexpected result based on previous studies with related Fcp1-deficient strains (Kobor *et al*, 1999). In the simplest scenario, the further loss of Fcp1 activity brought about *SUB1* deletion

would have been expected to further enhance CTD phosphorylation. But since the opposite was observed, that is, CTD phosphorylation actually decreased, we propose that Sub1 affects CTD phosphorylation in multiple ways. An intriguing possibility would be that Sub1 might influence Ssu72 phosphatase activity. Although Sub1 and Ssu72 are related in several ways, there is no evidence of an interaction, genetic or biochemical, between them (e.g., Wu *et al.*, 1999). But it is noteworthy that these two proteins, initially discovered by their similar interactions with TFIIB, have now each been shown to have activities related to both mRNA 3' end formation and CTD phosphorylation. Another possibility is that Sub1 can block CTD kinase activity, and biochemical data in mammals in fact support this possibility: PC4 has been shown to be a substrate-specific inhibitor of CTD phosphorylation by CDKs, including CDK7/Kin28 (Schang *et al.*, 2000). While additional studies are necessary to elucidate the exact mechanism(s), our work has provided strong evidence that Sub1 function involves control of CTD modifiers.

The ability of Sub1 to function as a transcription antiterminator likely involves more than its ability to influence CTD phosphorylation. We showed previously that Sub1 interacts with a region of Rna15 that appears to constitute a termination domain (Aranda and Proudfoot, 2001), and presented evidence that Sub1 downregulates this activity (Calvo and Manley, 2001). Although the underlying mechanism remains to be determined, this likely constitutes an important aspect of Sub1 antitermination activity. However, it is likely that Sub1 enhances elongation by another mechanism independent of CTD phosphorylation, which nonetheless involves Fcp1. Specifically, previous studies by Reinberg and colleagues, involving both biochemical experiments with human transcription factors and genetic experiments in yeast, indicate that Fcp1 possesses a positive elongation function that is independent of its phosphatase activity (Cho *et al.*, 1999; Mandal *et al.*, 2002). The reduced levels of Fcp1 detected in the absence of Sub1 provide at least a partial explanation for the observed defect in transcription elongation.

Materials and methods

Yeast strains

Genotypes of yeast strains used are as follows:

Δ sub1 MAT α or α ura3 trp1 ade2 leu2 his3 Δ sub1::URA3 (Calvo and Manley, 2001)

rna15-1 MAT α ura3 trp1 ade2 leu2 his3 rna15-1 (Calvo and Manley, 2001)

YOC03 MAT α sub1 Δ ::URA3 [HASUB1 TRP CEN] (this study)

YOC04a MAT α ura3 trp1 ade2 leu2 his3 [HARNA15 LEU2 CEN] (this study)

YOC04b MAT α rna15-1 [HARNA15 LEU2 CEN] (this study)

YOC05 MAT α rna15-1 sub1 Δ ::URA3 [HARNA15 LEU2 CEN] (this study)

YOC06 and YOC07 strains are SUB1KIN28HA and Δ sub1KIN28HA spores, respectively, constructed by crossing YSB756 (Komarnitsky *et al.*, 2000) with Δ sub1 (Calvo and Manley, 2001), and dissecting tetrads

YSB625 MAT α ura3 leu2 trp1 his3 lys2 can1 ceg1-250 (Rodriguez *et al.*, 2000)

YSB491 MAT α ura3 leu2 trp1 his3 ade2 ade3 can1 ceg1-250 (Cho *et al.*, 1998)

YF131 MAT α , ura3 leu2 his3 Δ 200 rpb3-(HA)₃::LEU2 (Komarnitsky *et al.*, 2000)

YSB756 MAT α ura3 leu2 trp1 his3 ade2 ade3 can1 kin28 Δ ::LEU2 [HAKIN28 TRP CEN] (Komarnitsky *et al.*, 2000)

YSB592 MAT α ura3 leu2 trp1 his3 ade2 ade3 can1 kin28 Δ ::LEU2 [HAKIN28-T17D TRP CEN] (Komarnitsky *et al.*, 2000)

YSB595 MAT α ura3 leu2 trp1 his3 ade2 ade3 can1 kin28 Δ ::LEU2 [HAKIN28-T162V TRP CEN] (Rodriguez *et al.*, 2000)

YSB609 MAT α ura3 leu2 trp1 his3 ade2 ade3 can1 kin28 Δ ::LEU2 [HAKIN28-K36A TRP CEN] (Rodriguez *et al.*, 2000)

YMK18 MAT α ura3 leu2 trp1 his3 ade2 can1 fcp1 Δ ::LEU2 [FCP1 URA CEN] (Kobor *et al.*, 1999)

YMK20 MAT α ura3 leu2 trp1 his3 ade2 can1 fcp1 Δ ::LEU2 [fcp1-1 (R250A, P251A) TRP CEN] (Kobor *et al.*, 1999)

YMK28 MAT α ura3 leu2 trp1 his3 ade2 can1 fcp1 Δ ::LEU2 [fcp1-3 (Δ 2-134) TRP CEN] (Kobor *et al.*, 1999).

We also generated new yeast strains by crossing rna15-1 and/or Δ sub1 mutants with ceg1-250, fcp1-1,3 or kin28-T17D, kin28-K36A and kin28-T162A mutants (see Results for details).

Yeast strain constructions and other genetic manipulations were performed by standard procedures (Guthrie and Fink, 1991). To obtain double mutants, the sub1 strain was crossed with the appropriate mutant and the corresponding zygotes were sporulated to obtain tetrads. Spores were dissected in selective medium to differentiate wild type, single and double mutants. Strains were grown at 25 and/or 37°C in either YPD (rich medium 1% yeast extract, 2% bactotryptone, 2% glucose) or YNB (minimal medium 0.67% yeast nitrogen base without amino acids, 2% glucose or 2% galactose/1% raffinose) supplemented with appropriate amino acids when necessary. Strains were transformed by the modified lithium acetate technique (Gietz *et al.*, 1992) and plasmids were maintained by growth in selective medium.

Western blot analyses

Whole-cell extracts were prepared for Western blot as described previously (Patturajan *et al.*, 1998). For experiments requiring heat shock, cells were grown at 30°C until OD₆₀₀ reached 0.8. The culture was split in two: half was diluted 1:1 with fresh prewarmed medium and then incubated at 37°C for 60 min, and the other half was diluted 1:1 using fresh medium at room temperature and then incubated at 30°C for 60 min. Blots were probed with different antibodies for detection of phosphorylated and nonphosphorylated RNAP IIo and a: the monoclonal antibody 8WG16 that recognizes an unphosphorylated CTD epitope, and the monoclonals H14 and H5 directed against phosphorylated CTD Ser5 and Ser2 epitopes, respectively (BabCO Covance). Monoclonal anti-HA (12CA5) was from Roche), monoclonal anti-Pgk was from Molecular Probes, and monoclonal anti-Cox3 and polyclonal anti-Fcp1 were gifts from Dr T Barrientos and J Greenblatt, respectively.

Northern blot analyses

Total RNA was prepared as described (Schmitt *et al.*, 1990); 10 μ g was separated on a 1.2% agarose gel and then transferred to a nylon membrane. A 1.3 kb FCP1 fragment amplified by PCR from pMK86 plasmid bearing FCP1 wt gene (Kobor *et al.*, 1999) was used as probe.

Chromatin immunoprecipitations

We performed ChIPs as previously described (Komarnitsky *et al.*, 2000; Schroeder *et al.*, 2000). All yeast strains were grown in minimal medium at 30°C, with the exception of rna15-1 HA-RNA15, which was grown at 37°C, to an OD₆₀₀ of 0.5–1.0. We used anti-HA antibody to immunoprecipitate HA-tagged Rpb3, Kin28, Rna15 and Sub1; 8WG16 or H5 to immunoprecipitate RNAP II; and anti-Fcp1 to immunoprecipitate Fcp1. Radiolabeled PCR products were analyzed on agarose gels (routinely 25% of input and 100% of immunoprecipitate) and quantified by PhosphorImager. Numbers below gels were calculated essentially as described previously (Kim *et al.*, 2004): signal intensities of immunoprecipitated PCR products were compared to those of the total input, and the ratio of values from each PCR product of transcribed genes to that of the nontranscribed HMR gene or an intergenic region on chromosome V was calculated. Therefore, numbers obtained are x-fold over HMR or V (background signal). Numbers on the y-axis of graphs are detailed in the corresponding figure legend. All ChIP assays were repeated 3–8 times, and in each case typical results are shown.

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