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Phosphorylation of Serine 2 within the RNA Polymerase II C-Terminal Domain Couples Transcription and 3′ End Processing

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Summary

The largest subunit of RNA polymerase II contains a unique C-terminal domain important for coupling of transcription and mRNA processing. This domain consists of a repeated heptameric sequence (YSPTSPS) phosphorylated at serines 2 and 5. Serine 5 is phosphorylated during initiation and recruits capping enzyme. Serine 2 is phosphorylated during elongation by the Ctk1 kinase, a protein similar to mammalian Cdk9/P-TEFb. Chromatin immunoprecipitation was used to map positions of transcription elongation and mRNA processing factors in strains lacking Ctk1. Ctk1 is not required for association of elongation factors with transcribing polymerase. However, in *ctk1*Δ strains, the recruitment of polyadenylation factors to 3′ regions of genes is disrupted and changes in 3′ ends are seen. Therefore, Serine 2 phosphorylation by Ctk1 recruits factors for cotranscriptional 3′ end processing in vivo.

Introduction

It has become increasingly clear that pre-mRNA processing in eukaryotic cells occurs cotranscriptionally. Coupling of these events is dependent upon the RNA polymerase II (RNAP II) carboxy-terminal domain (CTD) (Hirose and Manley, 2000; Proudfoot et al., 2002; Shatkin and Manley, 2000). The CTD consists of multiple repeats of the heptapeptide sequence YSPTSPS. In vivo, the CTD is phosphorylated predominantly at Serine 2 and Serine 5, and these phosphorylations are important for CTD function (Corden, 1990; West and Corden, 1995).

In *Saccharomyces cerevisiae*, four cyclin-dependent kinases have been identified as being important for transcription, and each one functions at a different point in the transcription cycle. The Kin28 (Cdk7) subunit of TFIIH phosphorylates the CTD on Serine 5 when polymerase is at the promoter. Serine 5 phosphorylation mediates recruitment and regulation of the mRNA capping enzyme guanylyltransferase (Cho et al., 1997, 1998; Ho and Shuman, 1999; Komarnitsky et al., 2000; Rodriguez et al., 2000; Schroeder et al., 2000; Yue et al., 1997). The Srb10 (Cdk8) kinase is associated with the Mediator complex and functions negatively to regulate initiation, perhaps by phosphorylation of the CTD (Hengartner et al., 1998) or by phosphorylating other transcription factors (Akoulitchev et al., 2000; Chi et al., 2001; Hirst et al., 1999; Nelson et al., 2003). The Bur1 kinase promotes transcription elongation, perhaps mediated by phosphorylation of the CTD or some other substrate (Keogh et al., 2003; Murray et al., 2001; Yao and Prelich, 2002).

The fourth CTD kinase is CTD kinase 1 (CTDK-I), which has also been implicated in transcription elongation. The catalytic subunit of CTDK-I is Ctk1 (Lee and Greenleaf, 1991). Bur1 and Ctk1 are equally similar in sequence to mammalian Cdk9, which has also been implicated in elongation (Price, 2000). Ctk1 associates with elongating RNAP II, where it phosphorylates the CTD on Serine 2 (Cho et al., 2001). The CTD phosphatase Fcp1 dephosphorylates this position during elongation, and the counteracting activities of Fcp1 and CTDK-I cause Serine 2 phosphorylation levels to increase as the polymerase moves further from the promoter (Cho et al., 2001).

Normal mRNA splicing and 3′ end processing are disrupted in mammalian cells expressing RNAP II lacking the CTD (McCracken et al., 1997). In yeast, splicing appears unaffected while polyadenylation becomes less efficient in the absence of the CTD (Licatalosi et al., 2002). Since elongating polymerase is primarily phosphorylated at CTD Serine 2, we proposed that this modification may recruit elongation, splicing, or polyadenylation factors during ongoing transcription (Cho et al., 2001; Komarnitsky et al., 2000). Experiments related to this hypothesis have produced mixed results. Greenleaf and colleagues isolated the polyadenylation factor Pti1 as a suppressor of a *CTK1* deletion (Skaar and Greenleaf, 2002). Several polyadenylation factors have been reported to bind the CTD in vitro, but binding has been seen to both to the nonphosphorylated and phosphorylated forms (Hirose and Manley, 2000; Proudfoot et al., 2002). Bentley and colleagues found that the Pcf11 polyadenylation factor bound efficiently in vitro to a Serine 2 phosphorylated CTD peptide but not to a Serine 5 phosphorylated peptide (Licatalosi et al., 2002). They also reported crosslinking of the Pcf11 and Fip1 polyadenylation factors to coding regions of transcribed genes. However, this crosslinking was not dependent upon the presence of the Ctk1 kinase, arguing against a requirement for Serine 2 phosphorylation in recruiting polyadenylation factors.

To directly test the hypothesis that Serine 2 phosphorylation of the CTD recruits elongation or polyadenylation factors, we used chromatin immunoprecipitation (ChIP) to compare the location of various factors along genes in wild-type and *ctk1*Δ cells. We saw no differences in elongation factors, indicating that their association with polymerase is not dependent upon CTD phosphorylation. In contrast, we find that phosphorylation of Serine 2 by Ctk1 is required for the recruitment of polyadenylation factors to the 3′ end of genes in vivo. Deletion of *CTK1* leads to changes in polyadenylation site choice, presumably due to the loss of cotranscriptional polyadenylation.

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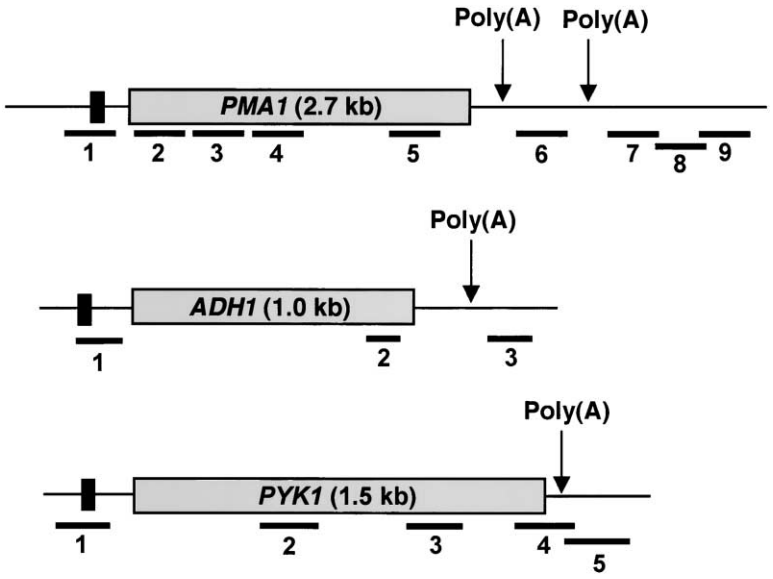

Figure 1. Schematic Diagram of the *PMA1*, *ADH1*, and *PYK1* Genes

For each gene, the open reading frame is represented by a gray box and the TATA box/promoter region by a black box. Arrows indicate the position of the major polyadenylation sites as reported previously (Kim et al., 2004). Bars below the gene show the positions of PCR products used in the chromatin immunoprecipitation and RT-PCR experiments. The numbers below each PCR fragment are used for identification in all later figures. See Supplemental Figure S1 (available online) for more details about primer positions and PCR product sizes.

Results

Ctk1 Is Not Required for the Recruitment of Elongation Factors In Vivo

To determine the positions of various elongation factors along genes in the presence and absence of CTD Serine 2 phosphorylation, ChIP was used as previously described (Kim et al., 2004). Three representative genes (*PMA1*, *ADH1*, and *PYK1*) were assayed because of their relatively high constitutive transcription rates and long open reading frames. The sites of polyadenylation and the PCR primer sets used in the ChIP assay are shown in Figure 1 and Supplemental Figure S1 (available online at http://www.molecule.org/cgi/content/full/13/1/67/DC1). Precipitation was via the TAP-tag (Puig et al., 2001; Rigaut et al., 1999), which we have previously shown to work very well in ChIPs (Kim et al., 2004). C-terminal TAP fusion proteins were constructed in strains containing or lacking Ctk1. Testing tagged Rpb3, we find that crosslinking is even and equal across genes even in the absence of Ctk1 (Figure 2). In agreement with our earlier results (Cho et al., 2001; Komarnitsky et al., 2000), deletion of Ctk1 does not affect CTD Serine 5 phosphorylation but severely reduces Serine 2 phosphorylation during elongation.

We previously found that elongation factors can be divided into two classes relative to their behavior at 3′ ends of genes. One class of elongation factors crosslinks throughout transcribed regions, including the transcribed sequences downstream of the cleavage/polyadenylation site (Kim et al., 2004). Spt4 and Spt5 form a complex that copurifies with RNAP II and other transcription factors (Gavin et al., 2002; Ho et al., 2002; Krogan et al., 2002; Lindstrom et al., 2003). DSIF, the mammalian homolog of this complex, was identified biochemically as affecting early steps in transcription elongation (Wada et al., 1998; Yamaguchi et al., 1999). Spt6 is associated with the Spt4/5-containing elongation complexes (Krogan et al., 2002; Lindstrom et al., 2003) and shares many genetic properties with Spt4/5. Spt16/Pob3 is the complex known as FACT in mammalian cells. All of these complexes are thought to help mediate transcription through chromatin. In wild-type cells, these factors show ChIP patterns matching that of RNAP II (Kim et al., 2004). When *CTK1* is deleted, the crosslinking patterns of these elongation factors are unaffected (Figures 3A and 3B). Therefore, Ctk1 is not required for the association of these factors with elongating polymerase.

The Paf (Squazzo et al., 2002) and TREX (Strasser et al., 2002) complexes also crosslink to coding regions of genes, but relative to RNAP II they show reduced crosslinking to transcribed regions downstream of the polyadenylation site (Kim et al., 2004). Dissociation of Paf and TREX at the 3′ ends of genes may help render the RNAP II competent for termination. Interestingly, levels of CTD Serine 2 phosphorylation also drop as the polymerase passes through the polyadenylation site. However, the recruitment of Paf and TREX complex components appear normal in the absence of Ctk1 (Figures 3C and 3D). The TFIIS elongation factor, which reactivates stalled RNAP II (Reines et al., 1996), has weak crosslinking to coding regions and drops at 3′ ends of genes. This factor was also unchanged in the absence of Ctk1 (data not shown). These ChIP results show that phosphorylation of CTD Serine 2 by Ctk1 is not required for the association of elongation factors with elongating RNAP II.

Ctk1 Is Required for Cotranscriptional Recruitment of Polyadenylation Factors In Vivo

To determine whether Serine 2 phosphorylation by Ctk1 affects the association between polyadenylation/cleavage factors and the RNAP II CTD in vivo, ChIP experiments using TAP-tagged polyadenylation/cleavage factors were carried out in both wild and *ctk1* deletion backgrounds. As shown in Figure 4, all except one of the polyadenylation/cleavage factors tested crosslinked very strongly near the polyadenylation sites of the genes. As previously observed (Komarnitsky et al., 2000), the hnRNAP protein Hrp1 (also known as polyadenylation factor CF IB) crosslinked throughout the coding regions of genes and to the 3′ end downstream of the polyade-

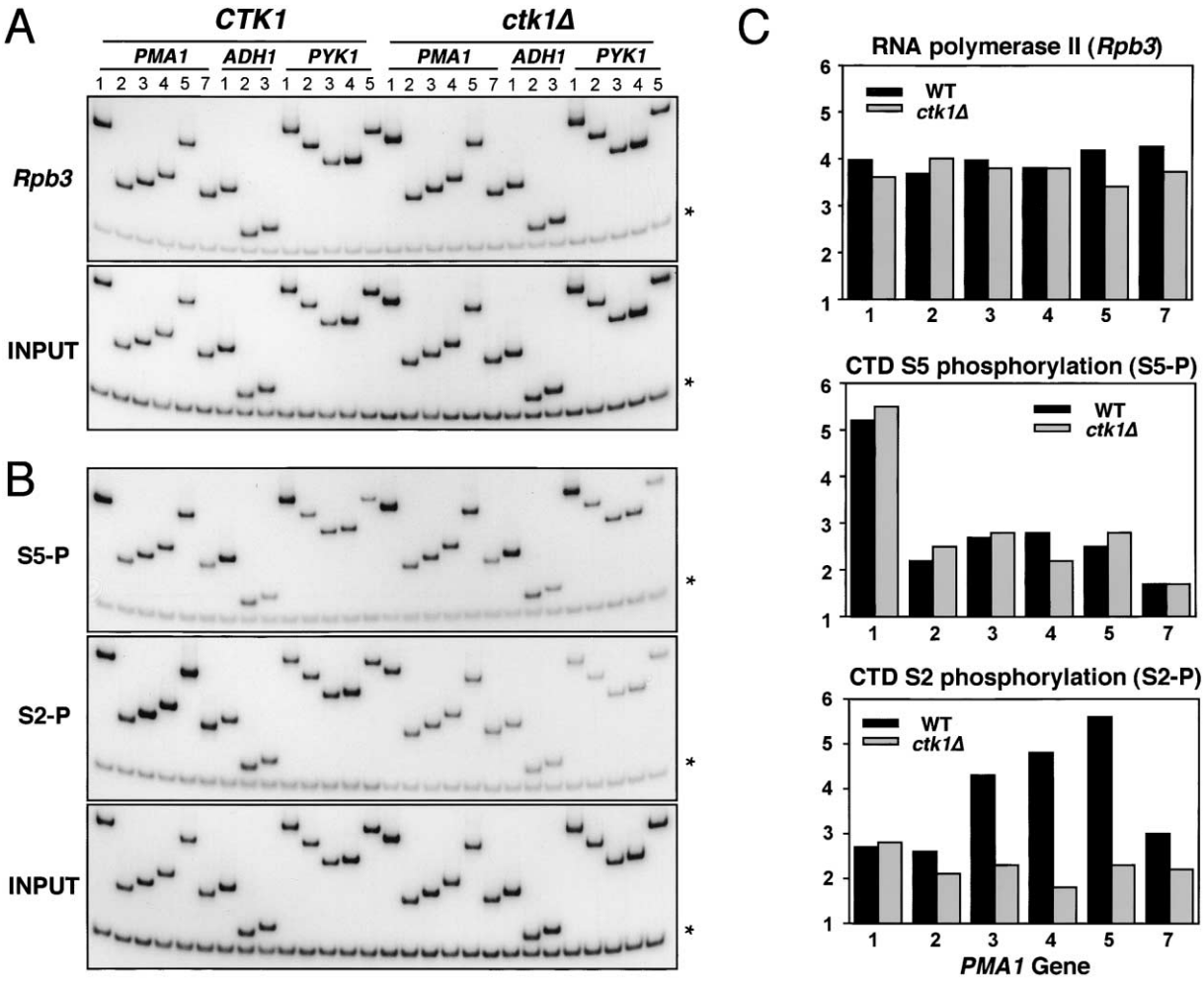

Figure 2. Transcription Elongation Is Normal in Cells Lacking Ctk1

(A) ChIP analysis of Rpb3 in cells containing or lacking Ctk1. The upper band in each lane is the PCR product for the indicated gene, with positions numbered as in Figure 1. The lower band (marked by asterisk) is the PCR product for a nontranscribed region that acts as a negative control for background.

(B) ChIP analysis of CTD phosphorylation levels. The monoclonal antibodies H14 and H5 were used to assay CTD Serine 5 and Serine 2 phosphorylation, respectively.

(C) Quantitation of the ChIP experiments in (A) and (B) for the *PMA1* gene. The *x* axis shows the number of the specific primer pair used in the PCR. The *y* axis shows the specific signal relative to the negative control (i.e., a ratio of one means the signal is that expected for background).

nylation site. Hrp1 is probably involved in packaging the mRNA properly for 3′ end processing and is not absolutely required for polyadenylation (Kessler et al., 1997; Minvielle-Sebastia et al., 1998). Hrp1 crosslinking was unaffected in the *ctk1*Δ background, indicating that its recruitment is not dependent on a phosphorylated CTD. In contrast, localization of the polyadenylation/cleavage factors to the 3′ ends of genes was severely reduced. These results indicate that phosphorylation of Serine 2 by Ctk1 is required for the efficient recruitment of polyadenylation/cleavage factors to the elongating RNAP II in vivo.

Ctk1 Deletion Affects Pre-mRNA 3′ Processing In Vivo

Since the absence of Ctk1 disrupts cotranscriptional recruitment of polyadenylation factors, *ctk1*Δ cells might be predicted to show defects in 3′ end processing. Indeed, Skaar and Greenleaf (2002) recently showed that *ctk1*Δ strains have altered polyadenylation site usage at the *RNA14* and *NCE102* genes. We confirmed the effect on *RNA14* using Northern blotting. The *RNA14* gene produces three transcripts (2.2, 1.5, and 1.1 kb) caused by processing at different poly(A) sites (Sparks and Dieckmann, 1998; see Figure 5A). Figure 5B shows that while the 1.5 kb transcript remained unaffected, the amount of the shortest transcript (1.1 kb) was markedly reduced and that of the 2.2 kb transcript was correspondingly increased. Interestingly, a very similar pattern is seen in cells carrying mutations in *RNA14* or *RNA15* (Mandart, 1998; Mandart and Parker, 1995).

Since the choice between multiple polyadenylation sites in *RNA14* can be affected by growth conditions (Sparks and Dieckmann, 1998), we wanted to examine the effects of *ctk1*Δ on a more typical gene. Therefore, RT-PCR was carried out using primer sets located near the two major poly(A) sites of the *PMA1* gene (Kim et

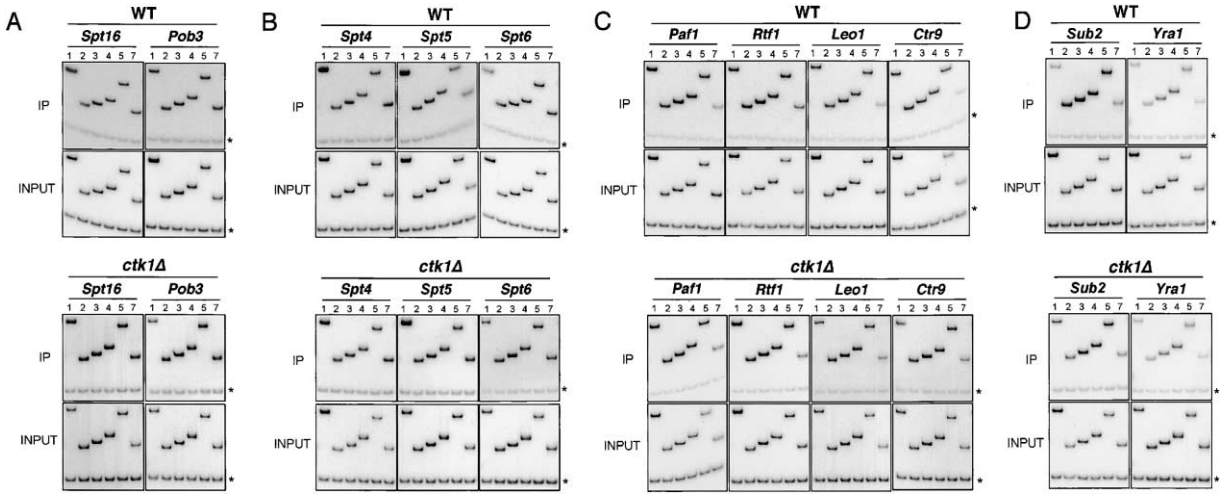

Figure 3. Ctk1 Is Not Required for the Recruitment of Elongation Factors In Vivo

ChIP analysis was carried out using *CTK1* (wt) or *ctk1*Δ deletion strains carrying the indicated TAP-tagged proteins. PCR analysis of immunoprecipitated chromatin was performed on the *PMA1* gene. In all cases, the patterns were similar to those seen in cells containing Ctk1. Quantitation showed no major differences (>2-fold) between crosslinking levels of factors. Similar results were obtained using other genes (data not shown).

(A) ChIP analysis of Spt16 and Pob3, the components of the FACT complex.

(B) ChIP analysis of Spt4, Spt5, and Spt6.

(C) ChIP analysis of components of the Paf complex.

(D) ChIP analysis of two components of the TREX complex.

al., 2004). In a *CTK1* strain, PCR products were obtained only with primers upstream of the most 3′ polyadenylation site (primers 5 and 6), indicating that termination and cleavage are very efficient. In contrast, the *ctk1*Δ strain yielded an additional product from a primer just downstream of the most 3′ polyadenylation site (set 7 in Figures 5C and 5D). The difference in signals is because primers 5 and 6 will amplify both correctly processed and readthrough mRNAs, while primer 7 only amplifies the readthrough mRNAs. Note that no signal was obtained with primer 9 located 400 bp further downstream, suggesting that transcription termination is still occurring between the two primers. Therefore, the *ctk1*Δ strain contains extended *PMA1* mRNAs. These may be formed due to either a pre-mRNA 3′ cleavage defect and/or a failure in transcription termination. Both of these possibilities are consistent with the loss of polyadenylation factors binding to the elongating polymerase as seen in the ChIP assays.

Ctk1 Deletion Does Not Affect Transcription Termination at *PMA1* In Vivo

The final step in the transcription process is termination, which is necessary to ensure that promoters are not perturbed by readthrough polymerases that have failed to terminate at upstream genes. Such transcriptional interference can occur in yeast where genes are closely spaced (Greger et al., 2000). To determine whether deletion of *CTK1* affects termination, ChIP was carried out using primer sets near the termination region of *PMA1* (numbers correspond to the primers sets indicated in Figure 1). *PMA1* is upstream of the *LEU1* gene but expression levels (copies/cell) and transcriptional frequency (mRNA/hr) of *PMA1* are over 10-fold higher than those of *LEU1* (Holstege et al., 1998). The observed signals in the intergenic region were completely due to *PMA1* transcription, since there was no signal for TBP or other basal transcription factors that would be indicative of *LEU1* promoter activity (data not shown).

In a *CTK1* strain, the level of Rpb3 drops between primer sets 7 and 8 (see the map in Figure 1 for the primer locations), indicating that this is the termination region (Figure 6). As previously observed (Kim et al., 2004), Serine 2 phosphorylation is decreased relative to Rpb3 immediately downstream of the polyadenylation site (primer set 7) but like Rpb3 drops to background levels further downstream (primer sets 8 and 9). Spt16 travels with RNAP II throughout the gene and crosslinks similarly to RNAP II during termination. The cleavage/polyadenylation factor Cft1 was recruited to elongating RNAP II near polyadenylation sites (Figure 4), but crosslinking also dropped along with RNAP II 3′ to the polyadenylation site (Figure 6). As in other experiments, essentially no crosslinking of Serine 2 phosphorylation or cleavage/polyadenylation factors was seen in a *ctk1*Δ deletion strain. Crosslinking of Rpb3 and Spt16 at the 3′ end of *PMA1* still dropped upon reaching the termination region (primer sets 8 and 9) even in the absence of Ctk1 and CTD Serine 2 phosphorylation (Figure 6). This agrees well with the RNA analysis (Figure 5) showing that extended transcripts are detected with primer 7 but not primer 9. Therefore, transcription termination at *PMA1* appears independent of Ctk1 activity despite the fact that cleavage is less efficient. This is surprising, since we found that termination at this gene is defective in an *rna14-1* mutant strain (Kim et al., 2004).

Discussion

The Ctk1 kinase is required for the bulk of CTD phosphorylation at serine 2 during elongation (Cho et al.,

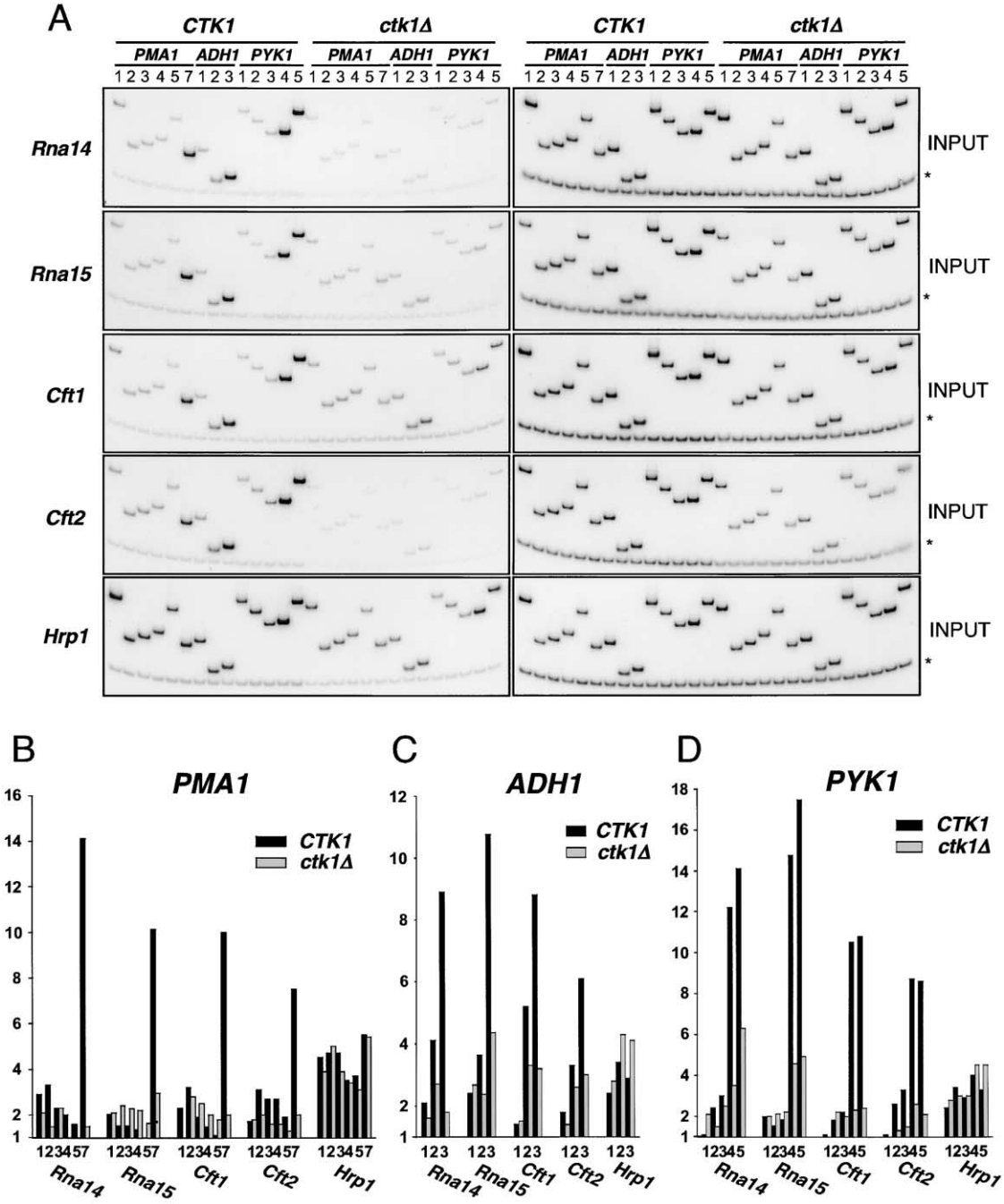

Figure 4. Ctk1 Is Required for Cotranscriptional Recruitment of Polyadenylation Factors In Vivo

(A) ChIP analysis was carried out with the indicated TAP-tagged proteins in both wild-type and Ctk1 deletion strains. PCR analysis of immunoprecipitated chromatin was performed on the *PMA1*, *ADH1*, and *PYK1* genes. The left panels show the ChIP signals while the right panels show the signals obtained with Input chromatin.

(B) Quantitation of results shown in (A).

2001). Somewhat surprisingly, Ctk1 is not essential, although deletion strains are cold sensitive and have several other mutant phenotypes. To see whether recruitment of various elongation and termination/polyadenylation factors to transcription complexes are dependent upon phosphorylation of CTD Serine 2, we compared their in vivo crosslinking patterns in wild-type and *CTK1* deletion strains.

RNAP II crosslinking is normal in strains lacking Ctk1 (Figure 2). This contrasts with cells treated with the inhibitor 6-azauracil or containing a mutant Bur1 kinase, where crosslinking of RNAP II drops off as the elongation complex moves further away from the promoter (Keogh et al., 2003). The ChIP results underscore the different functions of the two kinases: lack of Ctk1 leads to loss of CTD Serine 2 phosphorylation but does not obviously

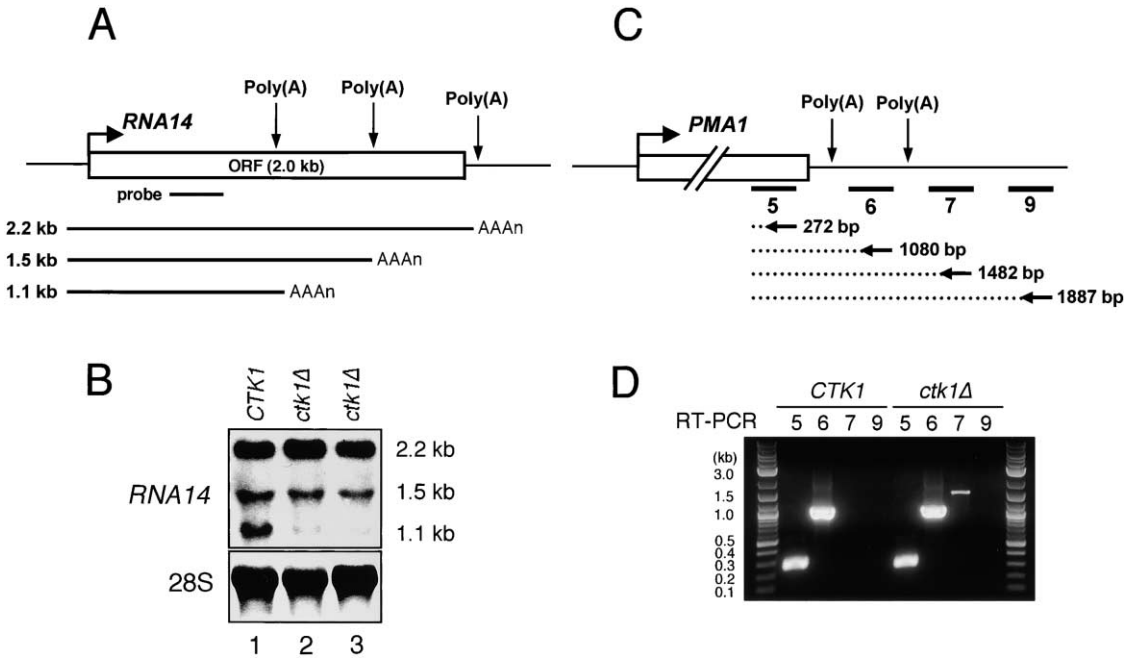

Figure 5. Deletion of *CTK1* Affects 3′ Processing of Pre-mRNA In Vivo

(A) Schematic representation of the *RNA14* gene and its transcripts. Arrows indicate the major poly(A) sites of RNA14 gene (Mandart and Parker, 1995). The position of the DNA fragment used as probe in the Northern blot analysis is indicated by a black bar.

(B) Northern blot analysis of *RNA14* mRNA isolated from the wild-type (lane 1, strain YSB726) or *ctk1*Δ deletion strains (lane 2, strain YSB854; lane 3, strain YF322). The size of each transcript is indicated in kilobases (kb). The bottom panel shows the 28S ribosomal RNA as a loading control.

(C) Schematic representation of *PMA1* termination region. Vertical arrows indicate the major poly(A) sites of *PMA1* gene. The relative position of each primer and the expected size of RT-PCR products are illustrated.

(D) Readthrough transcripts of *PMA1* are detected in a strain lacking Ctk1. Reverse transcription was carried out with the indicated antisense primers and then PCR was carried out using the same primer and the sense primer from fragment 5 (as shown in [C]). No products were observed when reverse transcriptase or primer was omitted during cDNA synthesis (data not shown).

affect elongation (this report; Cho et al., 2001), while a Bur1 mutant has defective elongation but apparently normal CTD phosphorylation (Keogh et al., 2003).

Strains lacking Ctk1 are weakly sensitive to 6-azauracil (Jona et al., 2001) and show genetic interactions with members of the Paf complex (Squazzo et al., 2002), TFIIS (Jona et al., 2001), and Spt4/5 (Lindstrom and Hartzog, 2001). Surprisingly, all elongation factors tested were recruited normally in the absence of Ctk1. Since CTD Serine 2 phosphorylation is not required for association of elongation factors, the interesting question remains as to what recruits them to elongation complexes. One likely candidate is certainly the RNA itself, but the fact that the elongation factors can be copurified with polymerase (Krogan et al., 2002; Lindstrom et al., 2003; Squazzo et al., 2002) suggests that other protein-protein interactions are also important.

In contrast to the elongation factors, localization of polyadenylation factors at the 3′ ends of genes is dependent upon Ctk1. Licatalosi et al. (2002) reported cross-

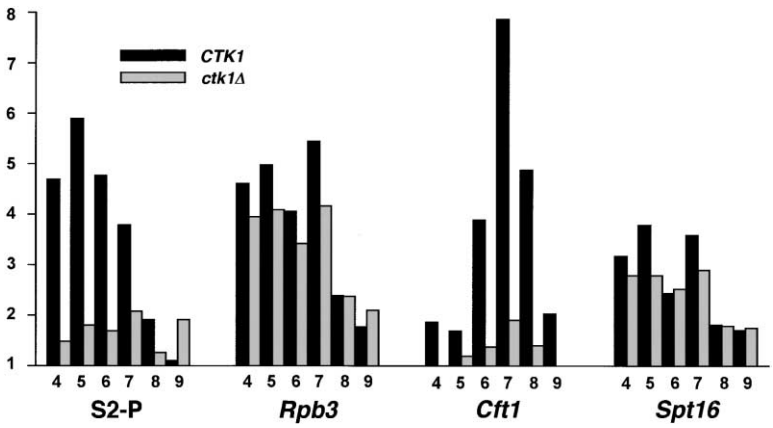

Figure 6. Transcription Termination at PMA1 Still Occurs in the Absence of Ctk1

ChIP analysis was carried out using strains carrying TAP-tagged Rpb3, Cft1, or Spt16. For analysis of Serine 2 phosphorylation, untagged strains were used. Wild-type (black bars) and *ctk1*Δ deletion (gray bars) strains were compared. The numbers below the bars correspond to PCR primer sets in the *PMA1* termination region as shown in Figure 1. ChIP signals were quantitated as described in the Experimental Procedures section.

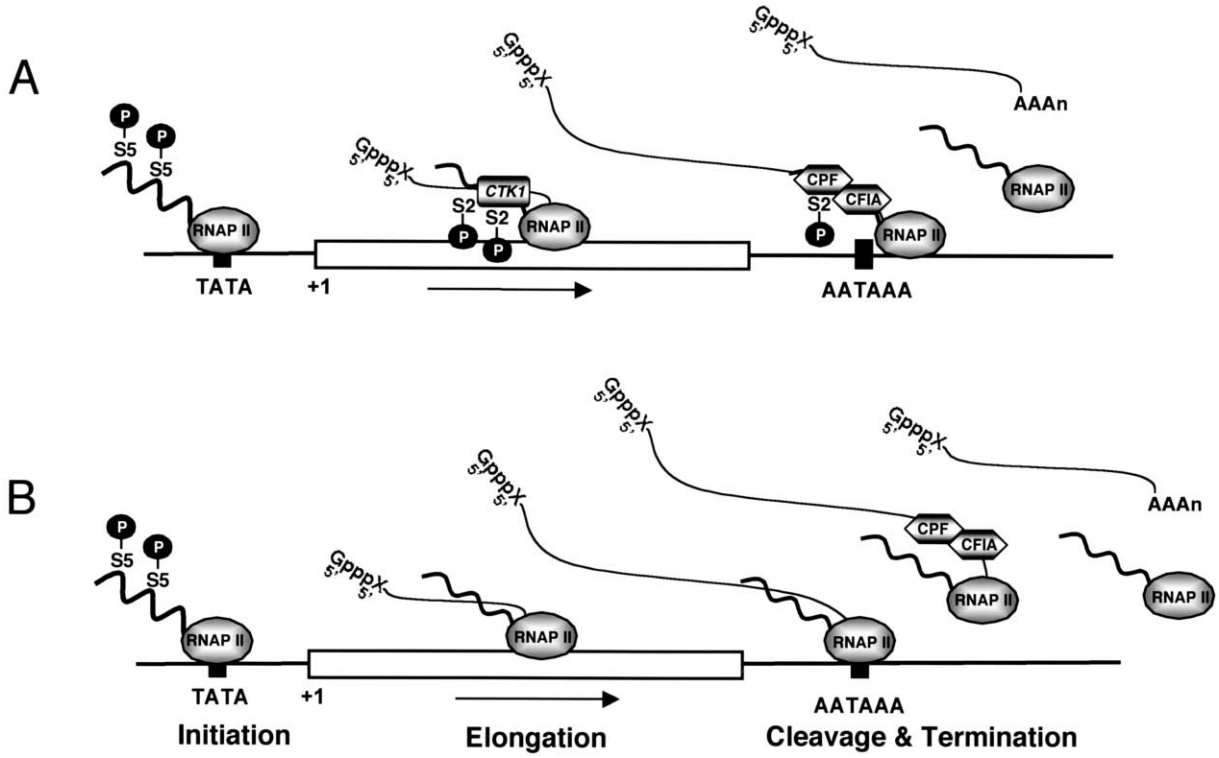

Figure 7. Two Possible Modes of Polyadenylation

Schematic diagram of (A) transcription-coupled and (B) uncoupled polyadenylation. See Discussion for details.

linking of two polyadenylation factors to coding regions of genes, but this was not dependent upon Ctk1. That study did not probe for factor crosslinking near the polyadenylation site. On some genes, we also see weak crosslinking to coding regions, but this signal is usually just above background and much weaker than the robust crosslinking at 3′ ends. This weak signal is not dependent upon Ctk1. Our ChIP results are more consistent with the finding that the Pcf11 polyadenylation factor binds in vitro to a CTD peptide phosphorylated at Serine 2 but not to a peptide phosphorylated at Serine 5 (Licatalosi et al., 2002).

The polyadenylation factors crosslink strongly only at the 3′ ends of genes, while Ctk1 and Serine 2 phosphorylation appear throughout the coding region (Cho et al., 2001; Komarnitsky et al., 2000). If the polyadenylation factors are recruited to polymerase via Serine 2 phosphorylation, one must ask why the two patterns don't perfectly overlap. There are two possible explanations. First, since Serine 2 phosphorylation levels are lowest during early elongation and increase with distance from the promoter (Cho et al., 2001; Komarnitsky et al., 2000), a certain threshold level of phosphorylation may be required before polyadenylation factors can bind with high affinity. This is unlikely to be the primary explanation, since polyadenylated transcripts range from a few hundred to several thousand nucleotides. A more likely model is that stable recruitment of polyadenylation factors requires a combination of two signals: the phosphorylation of CTD Serine 2 and the appearance of the polyA site sequences in the nascent mRNA (Figure 7A). Either feature alone could mediate some binding of polyadenylation factors, explaining why one can observe in vitro polyadenylation of pretranscribed precursor RNAs or direct protein-protein interactions between some polyadenylation factors and phosphorylated CTD. However, cooperative binding by these interactions would lead to synergistic recruitment of polyadenylation factors at the 3′ end where they function.

Why is Ctk1 not essential for viability in yeast (Lee and Greenleaf, 1991)? The simple answer is that polyadenylation must also occur in the absence of coupling to transcription, an idea strongly supported by several studies. It has been shown that RNAs generated by RNA polymerase I, RNA polymerase III, a viral polymerase, or T7 RNA polymerase can be cleaved and polyadenylated, although sometimes with reduced efficiency (Dower and Rosbash, 2002; Duvel et al., 2003; Fodor et al., 2000). In yeast, polyadenylation still occurs on mRNAs produced by RNAP II lacking a CTD, although efficiency and site selection are affected (Licatalosi et al., 2002). Purified yeast and mammalian systems lacking RNA polymerase II can also carry out polyadenylation in vitro independently of transcription, although the phosphorylated CTD stimulates this reaction (Hirose and Manley, 1998). In the absence of CTD-mediated coupling, yeast cells would be dependent upon noncoupled polyadenylation requiring the posttranscriptional interaction between the polyadenylation factors and the pre-mRNA (Figure 7B). This diffusion-dependent interaction could explain why *ctk1*Δ cells are cold sensitive and why this phenotype can be weakly suppressed by overexpres-

sion of the Pti1 polyadenylation factor (Skaar and Greenleaf, 2002).

Further supporting a role for Serine 2 phosphorylation in coupling between 3′ end processing and transcription, changes in polyA site selection are seen in *ctk1*Δ strains. This paper and a recent report from Skaar and Greenleaf (2002) show that polyadenylation of the *RNA14* transcript at the promoter-proximal site is lost in Ctk1 deletion strains. We also observe transcripts of the *PMA1* gene that extend beyond the normal polyadenylation sites, presumably due to delayed cleavage of the pre-mRNA. In the absence of coupling to transcription, the polyadenylation machinery presumably uses the highest affinity polyadenylation site(s) found on the mRNA. This would explain why most mRNAs are cleaved at the correct 3′ sites in yeast lacking Ctk1. However, targeting of polyadenylation factors to the transcription elongation complex allows the use of weaker polyadenylation sites. This may be because recruitment of the polyadenylation factors triggers an early transcription termination event, allowing weaker promoter-proximal cleavage/polyadenylation sites to be used before the polymerase transcribes stronger sites downstream. However, recruitment of polyadenylation factors to the Serine 2-phosphorylated CTD would also increase their local concentration and could promote binding to weaker polyadenylation sites even in the absence of any change in termination.

This model has important implications for alternative polyA site choice in many physiologically relevant systems. A classic example is the immunoglobulin loci, which produce a membrane-bound or secreted protein dependent upon whether polyadenylation occurs at an upstream site or further downstream of an alternative coding exon (for review, Edwalds-Gilbert et al., 1997). This choice can be influenced by the cellular concentration of a polyadenylation factor (Takagaki and Manley, 1998), and it has been proposed that the switch is in part determined by the differential affinity of the two sites for the polyadenylation machinery. However, another possibility not yet tested is that the two sites respond differently to changes in the level of transcription-coupled 3′ end processing.

In yeast, mutant forms of Rna14 or Rna15 lead to preferential use of downstream polyadenylation sites in *RNA14* and *ACT1* (Mandart, 1998; Mandart and Parker, 1995), similar to what is observed in the absence of Ctk1 (this report; Skaar and Greenleaf, 2002) or the CTD (Licatalosi et al., 2002). Since the shorter *RNA14* mRNAs do not produce functional protein, this alternative 3′ end formation may regulate the appropriate levels of Rna14 protein for transcription-coupled polyadenylation via a negative feedback loop.

Recently, a regulatory system that controls the timing of flowering has been discovered in *Arabidopsis*. The key regulatory proteins are a homolog of the yeast Pfs2 polyadenylation factor (FY) and an interacting WW-domain protein (FCA) (Simpson et al., 2003). The interaction between these two proteins leads to use of a promoter-proximal polyadenylation site in the FCA gene, producing a truncated mRNA and leading to downregulation of FCA as well as several other genes. Although it has been proposed that FCA binds to the mRNAs it regulates, many other WW-domain proteins are known to interact with the RNAPII CTD (Sudol et al., 2001). Another interesting possibility is that the FCA/FY complex is necessary for transcription-coupled polyadenylation at the promoter-proximal site, while at lower levels of the complex polyadenylation occurs primarily at the downstream site in a noncoupled manner.

Our results show that Ctk1 is required specifically for the coupling of transcription and mRNA polyadenylation. Lis and colleagues have reached similar conclusions in *Drosophila* (Ni et al., 2004 [this issue of *Molecular Cell*]). They found that inhibition of the P-TEFb/Cdk9 kinase in vivo using flavopiridol blocks phosphorylation of CTD Serine 2 and causes polyadenylation defects at the *hsp70* gene. Therefore, the recruitment of polyadenylation factors by CTD Serine 2 phosphorylation appears to be conserved throughout eukaryotes. It may be that this coupling is even more critical in higher eukaryotes, where transcripts are longer and often contain alternative 3′ ends.

Experimental Procedures

Yeast Strains

Strains used in this study are listed in Supplemental Table S1, available online. To generate TAP-tagged *ctk1*Δ strains, PCR was used to amplify TAP cassettes from the genomic DNA of tagged strains in the *CTK1* background (Kim et al., 2004; Krogan et al., 2002). The oligonucleotides used for PCR are available upon request. The resulting DNA fragments contained the 3′ end of the open reading frame fused to the TAP tag, a selectable marker, and additional downstream genomic sequence. The DNA fragments were transformed into the *ctk1*Δ strain (YSB854), and tagging was confirmed by immunoblotting for the TAP-tagged proteins as previously described (Puig et al., 2001).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitations and PCR reactions for TAP-tagged proteins were performed as described (Komarnitsky et al., 2000; Kim et al., 2004). Twenty-five PCR cycles were used, and control reactions show that amounts of product are linearly responsive to the input. Monoclonal antibodies H14 and H5 were purchased from Covance. For the Ser 2P and Ser 5P IPs, anti-mouse IgM antibodies coupled to agarose beads (Sigma) were bound to the H5 and H14 antibodies, respectively, and used to precipitate chromatin using modified binding and wash conditions developed by M.K. and M. Keogh. For both antibodies, binding was done overnight in FA lysis buffer (Komarnitsky et al., 2000) containing 150 mM NaCl. H5 immunoprecipitations were washed twice with the same binding buffer (FA lysis buffer with 150 mM NaCl), once with H5 wash buffer (2 mM Tris-HCl [pH 8.0], 0.02 mM EDTA, 50 mM LiCl, 0.1% Nonidet P-40, 0.1% sodium deoxycholate), and once with TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). For H14, beads were washed once with FA lysis buffer containing 275 mM NaCl, once with FA lysis buffer containing 500 mM NaCl, once with buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and once with TE. Elution and decrosslinking was as previously described (Komarnitsky et al., 2000).

PCR amplification was performed with primer pairs as shown in Figure 1 and Supplemental Figure S1 (available online). Oligonucleotide primer sequences are available upon request. Control experiments show that we can resolve signals from primer pairs 200 to 300 bp apart (Supplemental Figures S2 and S3, available online). PCR signals were quantitated by Fujix PhosphoImager. To control for amplification efficiency and label incorporation of different primers, the ratio of each gene-specific product to that of a nontranscribed region of chromosome V was calculated from the input sample signals. The signal for each specific gene primer in the immunoprecipitation was then divided by this ratio to convert the signal to normalized units. This value was then divided by the immunoprecipitation signal of the nontranscribed control product to de-

termine the fold enrichment of the ChIP signal over the background signal. This number is what appears on the *y* axis of the graphs. Because this number is a ratio, note that a value of 1 represents "no signal" (i.e., a signal equal to background).

RT-PCR and Northern Blotting

RNA was extracted from cells using the hot phenol method. For the RT-PCR experiment, first-strand cDNA preparation was performed using Superscript II reverse transcriptase (Invitrogen) and the indicated primer. PCR amplification was with Platinum Taq DNA polymerase (Invitrogen). Primer sets used were PMA1 (number 5): 5′-CTATTATTGATGCTTTGAAGACCTCCAG-3′ (sense) and 5′-TGCCC AAAATAATAGACATACCCCATAA-3′ (antisense); PMA1 (number 6): 5′-CAAGAAAGAAAAAGTACCATCCAGAG-3′ (antisense); PMA1 (number 7): 5′-GTAAATTTGTATACGTTCATGTAAGTG-3′ (antisense); PMA1 (number 9): 5′-CGTGATGAGTGAGTTAAGTTCTGCTG-3′ (antisense). PCR reactions were for 28 cycles (94°C, 1 min; 58°C, 1 min; 72°C, 1 min 50 s). Control reactions show that the PCR signals are linear with input. Note that primers 5 and 6 will amplify both properly processed and readthrough mRNAs, while primer 7 only amplifies readthrough transcripts.

For Northern analysis, total RNA was isolated as above from the indicated strains. Total RNA (25 μg) was separated on a 1% agarose gel and then transferred to a nylon membrane. Methylene blue staining of blots was performed to show that equal amounts of RNAs were loaded. The membrane was probed with body labeled *RNA14* probe followed by autoradiography. For the preparation of probe, *RNA14* sequences were PCR amplified from genomic DNA using two primers: 5′-GAGTCATACGCTAAGGTGAGAGAAG-3′ (sense) and 5′-CTGTAACTGCTGAATGTTTGAGTCTG-3′ (antisense). The PCR fragment was then labeled using random hexamer priming.

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References

Akoulitchev, S., Chuikov, S., and Reinberg, D. (2000). TFIIH is negatively regulated by cdk8-containing mediator complexes. Nature *407*, 102–106.

Chi, Y., Huddleston, M.J., Zhang, X., Young, R.A., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2001). Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. Genes Dev. *15*, 1078–1092.

Cho, E.J., Takagi, T., Moore, C.R., and Buratowski, S. (1997). mRNA capping enzyme is recruited to the transcription complex by phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev. *11*, 3319–3326.

Cho, E.J., Rodriguez, C.R., Takagi, T., and Buratowski, S. (1998). Allosteric interactions between capping enzyme subunits and the RNA polymerase II carboxy-terminal domain. Genes Dev. *12*, 3482–3487.

Cho, E.J., Kobor, M.S., Kim, M., Greenblatt, J., and Buratowski, S. (2001). Opposing effects of Ctk1 kinase and Fcp1 phosphatase at Ser 2 of the RNA polymerase II C-terminal domain. Genes Dev. *15*, 3319–3329.

Corden, J.L. (1990). Tails of RNA polymerase II. Trends Biochem. Sci. *15*, 383–387.

Dower, K., and Rosbash, M. (2002). T7 RNA polymerase-directed transcripts are processed in yeast and link 3′ end formation to mRNA nuclear export. RNA *8*, 686–697.

Duvel, K., Pries, R., and Braus, G.H. (2003). Polyadenylation of rRNA- and tRNA-based yeast transcripts cleaved by internal ribozyme activity. Curr. Genet. *43*, 255–262.

Edwalds-Gilbert, G., Veraldi, K.L., and Milcarek, C. (1997). Alternative poly(A) site selection in complex transcription units: means to an end? Nucleic Acids Res. *25*, 2547–2561.

Fodor, E., Mikulasova, A., Mingay, L.J., Poon, L.L., and Brownlee, G.G. (2000). Messenger RNAs that are not synthesized by RNA polymerase II can be 3′ end cleaved and polyadenylated. EMBO Rep. *1*, 513–518.

Gavin, A.C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J.M., Michon, A.M., Cruciat, C.M., et al. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature *415*, 141–147.

Greger, I.H., Aranda, A., and Proudfoot, N. (2000). Balancing transcriptional interference and initiation on the GAL7 promoter of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA *97*, 8415–8420.

Hengartner, C.J., Myer, V.E., Liao, S.M., Wilson, C.J., Koh, S.S., and Young, R.A. (1998). Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. Mol. Cell *2*, 43–53.

Hirose, Y., and Manley, J.L. (1998). RNA polymerase II is an essential mRNA polyadenylation factor. Nature *395*, 93–96.

Hirose, Y., and Manley, J.L. (2000). RNA polymerase II and the integration of nuclear events. Genes Dev. *14*, 1415–1429.

Hirst, M., Kobor, M.S., Kuriakose, N., Greenblatt, J., and Sadowski, I. (1999). GAL4 is regulated by the RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase SRB10/CDK8. Mol. Cell *3*, 673–678.

Ho, C.K., and Shuman, S. (1999). Distinct roles for CTD Ser-2 and Ser-5 phosphorylation in the recruitment and allosteric activation of mammalian mRNA capping enzyme. Mol. Cell *3*, 405–411.

Ho, Y., Gruhler, A., Heilbut, A., Bader, G.D., Moore, L., Adams, S.L., Millar, A., Taylor, P., Bennett, K., Boutilier, K., et al. (2002). Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. Nature *415*, 180–183.

Holstege, F.C., Jennings, E.G., Wyrick, J.J., Lee, T.I., Hengartner, C.J., Green, M.R., Golub, T.R., Lander, E.S., and Young, R.A. (1998). Dissecting the regulatory circuitry of a eukaryotic genome. Cell *95*, 717–728.

Jona, G., Wittschieben, B.O., Svejstrup, J.Q., and Gileadi, O. (2001). Involvement of yeast carboxy-terminal domain kinase I (CTDK-I) in transcription elongation in vivo. Gene *267*, 31–36.

Keogh, M.C., Podolny, V., and Buratowski, S. (2003). Bur1 kinase is required for efficient transcription by RNA polymerase II. Mol. Cell. Biol. *23*, 7005–7018.

Kessler, M.M., Henry, M.F., Shen, E., Zhao, J., Gross, S., Silver, P.A., and Moore, C.L. (1997). Hrp1, a sequence-specific RNA-binding protein that shuttles between the nucleus and the cytoplasm, is required for mRNA 3′-end formation in yeast. Genes Dev. *11*, 2545–2556.

Kim, M., Ahn, S.H., Krogan, N.J., Greenblatt, J., and Buratowski, S. (2004). Transitions in RNA polymerase II elongation complexes at the 3′ ends of genes. EMBO J., in press.

Komarnitsky, P., Cho, E.J., and Buratowski, S. (2000). Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev. *14*, 2452–2460.

Krogan, N.J., Kim, M., Ahn, S.H., Zhong, G., Kobor, M.S., Cagney, G., Emili, A., Shilatifard, A., Buratowski, S., and Greenblatt, J.F. (2002). RNA polymerase II elongation factors of Saccharomyces cerevisiae: a targeted proteomics approach. Mol. Cell. Biol. *22*, 6979–6992.

Lee, J.M., and Greenleaf, A.L. (1991). CTD kinase large subunit is encoded by CTK1, a gene required for normal growth of Saccharomyces cerevisiae. Gene Expr. *1*, 149–167.

Licatalosi, D.D., Geiger, G., Minet, M., Schroeder, S., Cilli, K., McNeil, J.B., and Bentley, D.L. (2002). Functional interaction of yeast pre-mRNA 3′ end processing factors with RNA polymerase II. Mol. Cell *9*, 1101–1111.

Lindstrom, D.L., and Hartzog, G.A. (2001). Genetic interactions of Spt4-Spt5 and TFIIS with the RNA polymerase II CTD and CTD

modifying enzymes in Saccharomyces cerevisiae. Genetics *159*, 487–497.

Lindstrom, D.L., Squazzo, S.L., Muster, N., Burckin, T.A., Wachter, K.C., Emigh, C.A., McCleery, J.A., Yates, J.R., 3rd, and Hartzog, G.A. (2003). Dual roles for Spt5 in pre-mRNA processing and transcription elongation revealed by identification of Spt5-associated proteins. Mol. Cell. Biol. *23*, 1368–1378.

Mandart, E. (1998). Effects of mutations in the Saccharomyces cerevisiae RNA14 gene on the abundance and polyadenylation of its transcripts. Mol. Gen. Genet. *258*, 16–25.

Mandart, E., and Parker, R. (1995). Effects of mutations in the Saccharomyces cerevisiae RNA14, RNA15, and PAP1 genes on polyadenylation in vivo. Mol. Cell. Biol. *15*, 6979–6986.

McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S.D., Wickens, M., and Bentley, D.L. (1997). The C-terminal domain of RNA polymerase II couples mRNA processing to transcription. Nature *385*, 357–361.

Minvielle-Sebastia, L., Beyer, K., Krecic, A.M., Hector, R.E., Swanson, M.S., and Keller, W. (1998). Control of cleavage site selection during mRNA 3′ end formation by a yeast hnRNP. EMBO J. *17*, 7454–7468.

Murray, S., Udupa, R., Yao, S., Hartzog, G., and Prelich, G. (2001). Phosphorylation of the RNA polymerase II carboxy-terminal domain by the Bur1 cyclin-dependent kinase. Mol. Cell. Biol. *21*, 4089–4096.

Nelson, C., Goto, S., Lund, K., Hung, W., and Sadowski, I. (2003). Srb10/Cdk8 regulates yeast filamentous growth by phosphorylating the transcription factor Ste12. Nature *421*, 187–190

Ni, Z., Schwartz, B.E., Werner, J., Suarez, J.-R., Lis, J.T. (2004). Coordination of transcription, RNA processing, and surveillance by P-TEFb kinase on heat shock genes. Mol. Cell *13*, this issue, 55–65.

Price, D.H. (2000). P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. Mol. Cell. Biol. *20*, 2629–2634.

Proudfoot, N.J., Furger, A., and Dye, M.J. (2002). Integrating mRNA processing with transcription. Cell *108*, 501–512.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods *24*, 218–229.

Reines, D., Conaway, J.W., and Conaway, R.C. (1996). The RNA polymerase II general elongation factors. Trends Biochem. Sci. *21*, 351–355.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. Nat. Biotechnol. *17*, 1030–1032.

Rodriguez, C.R., Cho, E.J., Keogh, M.C., Moore, C.L., Greenleaf, A.L., and Buratowski, S. (2000). Kin28, the TFIIH-associated carboxy-terminal domain kinase, facilitates the recruitment of mRNA processing machinery to RNA polymerase II. Mol. Cell. Biol. *20*, 104–112.

Schroeder, S.C., Schwer, B., Shuman, S., and Bentley, D. (2000). Dynamic association of capping enzymes with transcribing RNA polymerase II. Genes Dev. *14*, 2435–2440.

Shatkin, A.J., and Manley, J.L. (2000). The ends of the affair: capping and polyadenylation. Nat. Struct. Biol. *7*, 838–842.

Simpson, G.G., Dijkwel, P.P., Quesada, V., Henderson, I., and Dean, C. (2003). FY is an RNA 3′ end-processing factor that interacts with FCA to control the *Arabidopsis* floral transition. Cell *113*, 777–787.

Skaar, D.A., and Greenleaf, A.L. (2002). The RNA polymerase II CTD kinase CTDK-I affects pre-mRNA 3′ cleavage/polyadenylation through the processing component Pti1p. Mol. Cell *10*, 1429–1439.

Sparks, K.A., and Dieckmann, C.L. (1998). Regulation of poly(A) site choice of several yeast mRNAs. Nucleic Acids Res. *26*, 4676–4687.

Squazzo, S.L., Costa, P.J., Lindstrom, D.L., Kumer, K.E., Simic, R., Jennings, J.L., Link, A.J., Arndt, K.M., and Hartzog, G.A. (2002). The Paf1 complex physically and functionally associates with transcription elongation factors in vivo. EMBO J. *21*, 1764–1774.

Strasser, K., Masuda, S., Mason, P., Pfannstiel, J., Oppizzi, M., Rodriguez-Navarro, S., Rondon, A.G., Aguilera, A., Struhl, K., Reed, R., et al. (2002). TREX is a conserved complex coupling transcription with messenger RNA export. Nature *417*, 304–308.

Sudol, M., Sliwa, K., and Russo, T. (2001). Functions of WW domains in the nucleus. FEBS Lett. *490*, 190–195.

Takagaki, Y., and Manley, J.L. (1998). Levels of polyadenylation factor CstF-64 control IgM heavy chain mRNA accumulation and other events associated with B cell differentiation. Mol. Cell *2*, 761–771.

Wada, T., Takagi, T., Yamaguchi, Y., Ferdous, A., Imai, T., Hirose, S., Sugimoto, S., Yano, K., Hartzog, G.A., Winston, F., et al. (1998). DSIF, a novel transcription elongation factor that regulates RNA polymerase II processivity, is composed of human Spt4 and Spt5 homologs. Genes Dev. *12*, 343–356.

West, M.L., and Corden, J.L. (1995). Construction and analysis of yeast RNA polymerase II CTD deletion and substitution mutations. Genetics *140*, 1223–1233.

Yamaguchi, Y., Takagi, T., Wada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J., and Handa, H. (1999). NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA polymerase II elongation. Cell *97*, 41–51.

Yao, S., and Prelich, G. (2002). Activation of the Bur1-Bur2 cyclin-dependent kinase complex by Cak1. Mol. Cell. Biol. *22*, 6750–6758.

Yue, Z., Maldonado, E., Pillutla, R., Cho, H., Reinberg, D., and Shatkin, A.J. (1997). Mammalian capping enzyme complements mutant Saccharomyces cerevisiae lacking mRNA guanylyltransferase and selectively binds the elongating form of RNA polymerase II. Proc. Natl. Acad. Sci. USA *94*, 12898–12903.