UNIVERSITY OF CALIFORNIA, SAN DIEGO

The *S. cerevisiae* Nuclear Cap Binding Complex regulates RNA synthesis and processing through interactions with RNA Polymerase II

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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The Dissertation of Christina Chung is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chapter 2, in part is currently being prepared for submission for publication with T.L. Johnson as the corresponding author with the working title “The *S. cerevisiae* Nuclear Cap Binding Complex Interacts with RNA polymerase II to Regulate Nrd1 Complex Function." The dissertation author was the primary author on this manuscript.

PREFACE

This thesis was prepared posthumously and represents a significant portion of the work carried out by Christina Chung during her graduate studies.

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ABSTRACT OF THE DISSERTATION

The *S. cerevisiae* Nuclear Cap Binding Complex regulates RNA synthesis and processing through interactions with RNA Polymerase II

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Professor Tracy Johnson, Chair

The nuclear Cap Binding Complex (CBC), which consists of a large subunit CBP80 and a small subunit CBP20, is structurally conserved from yeast to mammals. The complex has been implicated in multiple steps in mRNA processing including mRNA stability, pre-mRNA splicing, and mRNA export. The research described here provides evidence that the CBC plays a novel role in transcription through interactions with the RNA polymerase II (RNAPII), particularly through the C-terminal domain of the largest subunit (CTD). These interactions appear at least partially RNA independent and occur during active transcription. Although the CBC does not possess kinase or phosphatase activity, it does appear to affect CTD phosphorylation. One effect of this CBC-CTD interaction is regulation of the Nrd1 termination complex activity in

attenuation of the IMP dehydrogenase gene *IMD2* and its constituent cryptic unstable transcripts (CUTs). The involvement of the CBC with the RNA from capping to export and with RNAPII places it in an ideal position to provide regulatory feedback from the nascent RNA and RNA processing factors to the transcriptional machinery.

Chapter 1

Introduction: The yeast cap binding complex and RNA polymerase II roles in gene expression

The Cap-Binding Complex

The cap-binding complex (CBC) is a heterodimeric complex consisting of a large subunit, Cbp80, and a small subunit, Cbp20. This complex is conserved from yeast to humans, and has been shown in both yeast and mammals to bind to the 5' m^7G cap of nascent pre-mRNAs (Mazza, Segref et al. 2002). This complex is one of the earliest to contact the pre-mRNA, is involved in proper 3' processing of the pre-mRNA and export of the pre-mRNA from the nucleus (reviewed in Lewis and Izaurralde 1997), as well as facilitating RNA stability (Hosoda, Kim et al. 2005; Das, Das et al. 2006). The CBC has also been shown to play a role in splicing: it recruits the U1 snRNP to the 5' splice site to form the commitment complex (the mammalian E complex) (Lewis, Gorlich et al. 1996; Du and Rosbash 2001) and appears to play a role in mediating rearrangements of the spliceosome following U1 snRNP recruitment (O'Mullane and Eperon 1998). Perhaps most relevant, the CBC is required for proper co-transcriptional recruitment of the spliceosome (Görnemann, Kotovic et al. 2005).

While a clear role for the CBC in transcription initiation and/or elongation has yet been described, the literature provides enticing hints of such a role. The large subunit of the CBC, *CBP80*, was originally isolated in yeast as *GCR3* in a screen for factors affecting glycolytic gene expression (Uemura and Jigami 1992). Two other genes, *GCR1* and *GCR2*, were isolated with very similar phenotypes to *GCR3*/*CBP80* and have been shown to function as a transcriptional activator complex (Clifton, Weinstock et al. 1978; Clifton and Fraenkel 1981; Kawasaki and Fraenkel 1982; Holland, Yokoi et al. 1987; Uemura and Jigami 1992). *CBP80* mutations in yeast also suppress temperature sensitive mutations of *HPR1* (Uemura, Pandit et al. 1996), a member of the THO transcription elongation complex; other suppressors include RNAPII subunits and transcription factors (Fan and Klein 1994; Santos-Rosa and Aguilera 1995; Fan, Cheng et al. 1996). Several studies in our lab demonstrate that CBC deletion mutants grow slowly in the presence of the drug 6-azauracil (6AU) (Riles, Shaw et al. 2004). Assaying for sensitivity to 6AU is a common diagnostic technique for strains with transcription elongation defects.

The Carboxy-Terminal Domain of RNAPII

The carboxy-terminal domain of RNAPII (CTD) consists of a series of heptapeptide repeats of YSPTSPS conserved from yeast (with 26 repeats) to

humans (with 52 repeats) that undergo cycles of phosphorylation and dephosphorylation during transcription. These changes that occur on serine-2 and serine-5 of the heptapeptide repeats correlate to changes in distinct phases of transcription (reviewed in Palancade and Bensaude 2003; Phatnani and Greenleaf 2006). The polymerase enters the PIC with the CTD largely unphosphorylated, as transcription initiation (early transcription) begins, an increase in serine-5 phosphorylation is seen, and as transcription progresses into elongation an increase in serine-2 phosphorylation occurs (Palancade and Bensaude 2003).

A number of kinase complexes and phosphatases are involved in setting up these cycles of phosphorylation and dephosphorylation, and early work in our lab with *CBC* deletion mutants showed synthetic lethality with deletion of subunits from two yeast kinase complexes, Ctk1/2/3 and Bur1/2. Both of these kinase complexes are equally orthologous to the mammalian transcription elongation factor and CTD kinase complex, P-TEFb, and have also been implicated in transcription elongation (Cho, Kobor et al. 2001; Murray, Udupa et al. 2001; Keogh, Podolny et al. 2003). I have demonstrated that genetic interactions can also be observed between the CBC and the CTD repeats. Deletion of the CBC subunits rendered a full-length CTD necessary for wild-type growth, and truncation of the CTD to 10 repeats, while normally viable, was lethal in the CBC deletion background. This suggested that there

exists an overlap in function between the CBC and the CTD, possibly due to involvement of both in the coordination of transcription and RNA processing.

The CTD has been suggested to play a role in coordinating RNA processing and transcription. Because of its unique series of heptapeptide repeats, the idea of the CTD as a docking platform has become prominent. Both transcription factors and RNA processing factors have been found to "dock" at the CTD; notable among them are the capping enzymes (Cho, Takagi et al. 1997; McCracken, Fong et al. 1997; Yue, Maldonado et al. 1997), the SR and SR-like proteins in mammals (reviewed in (Corden and Patturajan 1997)), the yeast splicing factor Prp40 (Morris and Greenleaf 2000), and the Spt4/Spt5 transcription complex (known as DSIF in mammals) (Lindstrom and Hartzog 2001). Additionally, the CTD can be reversibly modified by phosphorylation to create specific binding sites for this diverse group of CTD interacting factors. My findings of genetic interactions between the CBC and both the CTD and its modifying enzymes have led me to explore the physical interactions between the CBC and the RNAPII, specifically the CTD. Furthermore, the studies described here explore a model in which the CBC associates with the CTD of RNAPII to facilitate transcription elongation.

Chapter 2

The *S. cerevisiae* Nuclear Cap Binding Complex Interacts with RNA polymerase II to Regulate Nrd1 Complex Function

ABSTRACT

In recent years, there has been accumulating evidence of cryptic transcription throughout the genome, some of which has important regulatory consequences. One class of these transcripts, CUTs (Cryptic Unstable Transcripts) is particularly important in regulation of genes that allow cells to rapidly respond to changes in environmental conditions. A complex that plays a critical role in regulation of these transcripts is the Nrd1-Sen complex. While it appears that the complex is recruited to the transcriptional machinery, in part, via interactions between Nrd1 and the c-terminal domain of RNAPII (CTD), it is unclear what factors act upstream of the Nrd1 complex. Here we show that the nuclear cap binding complex (CBC) regulates the expression of the *IMD2* CUTs, and we further show that the CBC regulates the activity of the Nrd1 termination complex through interactions with RNA polymerase II. This RNAPII-CBC interaction is not abolished by treatment with RNase and is not dependent on Nrd1's CTD interaction domain. We further show that the CBC mediates changes in CTD phosphorylation that have been shown to alter Nrd1 recruitment to CUTs. We propose a model whereby the CBC, through

alterations in CTD phosphorylation, regulates the Nrd1 termination complex function.

INTRODUCTION

Eukaryotic pre-messenger RNAs undergo extensive processing before the mature message can serve as a proper template for protein synthesis. The newly synthesized RNA is capped with a 5' 7-methylguanosine; non-coding intron sequences are removed through the process of RNA splicing; and a poly(A) tail is added to the 3' end of the message. While each of these processes has been characterized as a distinct biochemical reaction, there is evidence showing that these processes are spatially and temporally coupled and that each of these reactions can occur co-transcriptionally (for review see (Maniatis and Reed 2002; Aguilera 2005)). The factors involved in coupling these processes, other than RNA polymerase II, and the mechanism by which these interactions are coupled are not well understood.

One complex that has been implicated in multiple RNA processing steps is the heterodimeric protein complex known as the nuclear cap binding complex (CBC). This complex consists of a large and small subunit, Cbp80 and Cbp20 respectively, which show sequence conservation from yeast to mammals. Upon synthesis of the 5' cap, pre-mRNAs are co-transcriptionally bound to this complex, and the CBC remains bound to the mature RNA as it exits the nucleus. During its association with the RNA transcript, the yeast CBC has been shown to participate in RNA splicing (Lewis, Gorlich et al. 1996; Fortes, Bilbao-Cortes et al. 1999) and in RNA surveillance in association with the nuclear exosome (Das, Butler et al. 2003; Culbertson and Neeno-

Eckwall 2005). The mammalian CBC has been shown to participate in 3' end processing, particularly the cleavage step (Flaherty, Fortes et al. 1997; Wong, Qiu et al. 2007). Most recently, the yeast CBC has been shown to suppress the use of weak termination sites (Wong, Qiu et al. 2007).

However, there is little evidence that the CBC is able to also play a direct role in regulating transcription. The mammalian CBC co-immunoprecipitates with RNAPII (Lejeune, Ishigaki et al. 2002) as well as the negative elongation factor (NELF) (Narita, Yung et al. 2007). Deletion of either CBC subunit in yeast has also been found to result in growth sensitivity to the drug 6-azauracil (Wong, Qiu et al. 2007), which is a phenotype commonly attributed to strains with transcription elongation defects, and has been connected to difficulty in transcribing the IMP dehydrogenase gene *IMD2* either because of general defects in transcription elongation or misregulation of regulatory transcripts synthesized from regions upstream of the *IMD2* gene (Kopcewicz, O'Rourke et al. 2007; Jenks, O'Rourke et al. 2008).

The *IMD2* gene locus is of particular interest to us in that it consists of several highly regulated transcripts: an *IMD2* mRNA that is transcribed under low GTP conditions to produce the IMP dehydrogenase; and a second set of transcripts that are cryptic unstable transcripts (CUTs) transcribed under high GTP conditions from an initiation site upstream of the *IMD2* mRNA. Detailed examination of *IMD2* transcription can distinguish between the 6AU sensitivity caused by general elongation defects and 6AU sensitivity caused by

misregulation of the *IMD2* CUTs, which are obviously distinct regulatory mechanisms.

CUTs were originally identified as short RNAPII transcripts stabilized by the loss of the nuclear exosome factor, Rrp6. These cryptic transcripts are terminated by the Nrd1 complex (Nrd1-Nab3-Sen1) in conjunction with the TRAMP complex (Trf4-Air1/Air2-Mtr4 polyadenylation complex) (Wyers, Rougemaille et al. 2005; Davis and Ares 2006).

The Nrd1 termination complex is also involved in termination of snRNAs and snoRNAs (Steinmetz and Brow 1996; Ursic, Himmel et al. 1997; Rasmussen and Culbertson 1998; Steinmetz, Conrad et al. 2001), but its role in termination of CUTs has recently been shown for the *IMD2* gene. The Nrd1 complex has also been found to associate with RNAPII and the nuclear exosome (Vasiljeva and Buratowski 2006), as welll as subunits of the CBC. These interactions further suggest a role for the CBC in regulatory activities involving these factors.

The Nrd1 protein contains a domain that interacts with the c-terminal domain of the largest subunit of RNAPII (CTD) (Yuryev, Patturajan et al. 1996). The CTD, a conserved domain found from yeast to mammals, consists of multiple heptapeptide repeats of YSPTSPS that undergoes cycles of phosphorylation and dephosphorylation on its serines in a manner correlated to the different stages of transcription. Hence, the CTD is thought to produce a changing platform for transcription factors and RNA processing factors to bind

and interact. The Nrd1 CTD interaction domain (CID) interacts specifically with the serine-5 phosphorylated form of the CTD (Vasiljeva, Kim et al. 2008), which is found early during transcription initiation. The Nrd1-CTD interaction appears to require a very specific ratio of serine-2 to serine-5 phosphorylations and is crucial for function of the Nrd1 complex in termination of CUTs (Gudipati, Villa et al. 2008).

Because of the physical interactions between the Nrd1 complex and the CBC, we examined the role of the CBC in termination of the *IMD2* CUTs and find that it shares similar phenotypes with a Nrd1 mutant lacking the CID. This led us to examine whether the CBC may act upstream of Nrd1 to regulate its activity. Here we report that the CBC can interact with RNAPII independent of the Nrd1 CID and, in fact, Nrd1 recruitment to genes is altered by the loss of CBC subunits. The CBC interacts genetically with the CTD of RNAPII as well as CTD kinases (Hossain and Johnson, submitted), leading to a model in which interactions between the CBC and RNAPII facilitate Nrd1 activity and its regulation of CUTs.

The interactions we have identified between the CBC and RNAPII are likely to have important consequences for coordination of transcription with RNA processing beyond its effects on the Nrd1 termination complex function. The CBC is in an ideal position to provide feedback about the nascent RNA to the transcriptional machinery and RNA processing factors.

RESULTS

The CBC attenuates transcription of *IMD2*.

Deletion of either CBC subunit results in sensitivity to the drug 6-azauracil (6AU) (Wong, Qiu et al. 2007). 6AU depletes intracellular pools of GTP, and sensitivity to the drug is a hallmark of cells deficient in transcription elongation, such as deletion of TFIIS (*dst1Δ*) as well as in truncation mutants of the CTD of Rpb1. This 6AU-sensitivity observed in these strains is caused, at least in part, by difficulty transcribing the IMP dehydrogenase gene, *IMD2*. To determine if the CBC shows phenotypes typical of transcription elongation factors, we analyzed *IMD2* RNA by Northern in the presence of 6AU. In strains lacking *CBP80* or *CBP20*, the dramatic decrease in *IMD2* transcript observed with elongation mutants is (Figure 1A, lanes 6 and 7 as compared to lane 8 and Figure 1B, lane 6).

IMD2 is regulated by expression of CUTs upstream of the normal ORF (Davis and Ares 2006). These CUTs require a functional Sen1 helicase, a component of the Nrd1 complex, for proper termination under uninduced conditions (Steinmetz, Ng et al. 2006). Since the CBC has been shown to be a component of the Nrd1 complex (Vasiljeva and Buratowski 2006), and the CBC activity can affect termination site choice (Wong, Qiu et al. 2007) we addressed whether the CBC is required for termination of the *IMD2* CUTs.

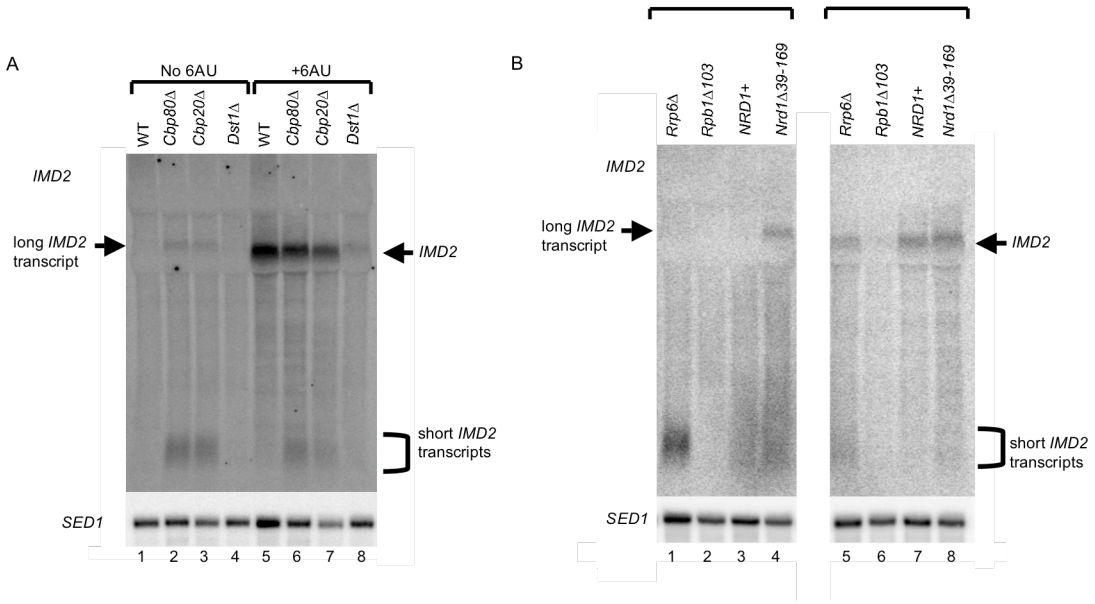


FIGURE 1. CBC mutants exhibit an *IMD2* attenuation defect. (A) Northern blot of *IMD2* transcripts in WT, *cbp80Δ*, *cbp20Δ*, and *dst1Δ* strains. Bottom panel is the same blot stripped and re-probed for *SED1* as a loading control. (B) Northern blot of *IMD2* transcripts in *rrp6Δ*, *rpb1Δ103* (Rpb1 truncated to 10 CTD repeats), *NRD1+* (*nrd1Δ* carrying a wild type copy of *NRD1* on a plasmid), and *nrd1Δ39-169* strains. Bottom panel is the same blot stripped and re-probed for *SED1* as a loading control.

Under uninduced conditions a *sen1* mutant produces a long *IMD2* transcript in which the 5' termination site is read-through, rather than producing short rapidly degraded CUTs (Steinmetz, Ng et al. 2006). The long *IMD2* transcript is also produced when the CBC subunits are deleted (Figure 1A compare lane 1 to lanes 2 and 3). This is the same product generated in cells carrying a Nrd1 mutant lacking the CTD interaction domain (*nrd1Δ39-169*) (Figure 1B, lanes 3 and 4), suggesting that the CBC and the Nrd1 CID both are involved in dictating the Nrd1 complex activity. *nrd1Δ39-169* is still able to trigger termination and degradation, but we find that a double mutant with a *CBP80* deletion and lacking the Nrd1 CID does not have an exacerbated read-through *IMD2* CUTs phenotype (data not shown). This suggests to us that the Nrd1 CID and the CBC have an overlapping function in dictating Nrd1 complex termination.

In the presence of 6AU, we observe nearly wild type levels of full length *IMD2* transcript in either the CBC mutant strains (Figure 1A, lanes 6 and 7) or the *nrd1Δ39-169* mutant (Figure 1B, compare lanes 7 to 8), demonstrating that neither the CBC nor the CTD interaction domain (CID) of Nrd1 is defective in normal *IMD2* transcription. These results are consistent with previous studies suggesting that the 6AU sensitivity of CBC is not due to direct effects on transcription elongation (Wong, Qiu et al. 2007). The 6AU sensitivity is likely caused by the presence of the *IMD2* CUTs or the read-through transcript.

While Nrd1 and the CBC appear to share common activities that affect transcription of *IMD2* and its CUTs, there are also differences in the *IMD2* transcripts produced in the CBC deletion strains compared to the Nrd1 complex mutants. In strains deleted of the CBC, the short *IMD2* transcripts normally produced by Nrd1 termination and degraded by the nuclear exosome are produced under both uninduced and induced conditions (Figure 1A, lane 2-3 and 6-7 compared to lanes 4 and 8). These short *IMD2* transcripts are similar to those produced in the *rrp6Δ* strain (Figure 1B, lane 1) suggesting that the cells lacking CBC subunits may be deficient in nuclear exosome activity. This may not be surprising since the CBC has also been found to be required for RNA degradation by the exosome. Since the CBC behaves similarly to the Nrd1 CID mutant in production of a long read-through *IMD2* transcript, we considered the possibility that these factors are involved in related activities.

The CBC affects recruitment of Nrd1.

The CBC is thought to be present on the RNA shortly after the cap is attached. So the CBC is expected to be associated with the transcribing complex prior to Nrd1 complex association. To address whether the CBC might change recruitment of Nrd1 during transcription, chromatin immunoprecipitation (ChIP) was performed. Under uninduced conditions, where we expect to see an effect of *cbcΔ*, the ChIP signal on *IMD2* is too low

to detect. So we chose to look at a highly transcribed gene to which Nrd1 has previously been found to associate, *PMA1* (Steinmetz, Ng et al. 2006). Loss of either CBC subunit results in loss of the peak of Nrd1 recruitment at the 5' end of *PMA1* (Figure 2). Since the Nrd1 complex terminates a number of short RNAs including CUTs, snoRNAs, and snRNAs which all terminate a few hundred nucleotides from the initiation start site, Nrd1 occupancy near the 5' ORF primer set is not unexpected. This result suggests that Nrd1 may generally be present at the 5' end of genes in a CBC dependent manner placing it in an optimal position to terminate transcription when the appropriate signals are present.

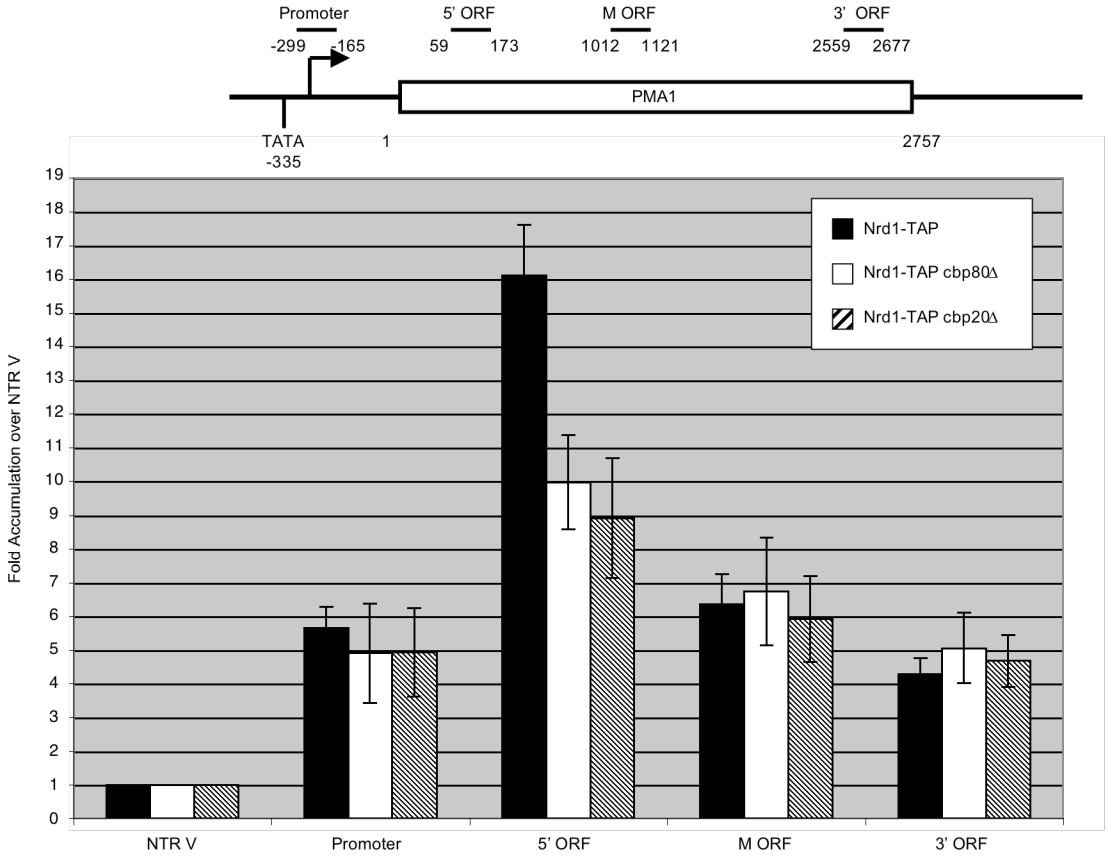


FIGURE 2. CBC plays a role in Nrd1 interactions with the transcribing machinery. ChIP of Nrd1-TAP in the presence or absence of Cbp80 and Cbp20 on *PMA1*.

CBC associates with RNAP II in a complex that requires both CBC subunits, is RNase resistant and Nrd1 CID independent.

The CBC role in Nrd1 recruitment and the similarity of its *IMD2* transcription phenotype to the Nrd1 CID mutant suggest that the CBC may itself be interacting with the transcriptional machinery. To address this we examined whether a physical interaction exists between the CBC and the RNAPII. Under mild salt conditions TAP-tagged CBC subunits co-immunoprecipitate with the large subunit of RNAPII, Rpb1 (Figure 3A, lanes 3 and 4), when compared to mock co-immunoprecipitation carried out in an untagged wild-type strain (Figure 3A, lane 1). This co-immunoprecipitation interaction is comparable to that observed between Rpb1 and the transcription elongation factor Spt5, a factor which is known to interact directly with the phosphorylated form of the CTD (Figure 3A, lane 5) (Lindstrom and Hartzog 2001). Not surprisingly the CBC interaction with RNAPII is weaker, likely because the CBC plays a dynamic role in the cell that does not require all of the complex to be associated with the transcribing machinery and nascent transcript at all times.

Previous studies have shown that co-immunoprecipitations can sometimes results from interactions that occur in solution after cells are lysed (Mili and Steitz 2004). To be sure that we were looking at actual *in vivo* interactions between the CBC and RNAPII and not interactions formed in solution, a brief formaldehyde cross-linking step was added. Furthermore, to

ensure that the interactions observed were not due to DNA-CBC interactions a sonication step was added prior to immunoprecipitation. The CBC remains clearly detectable in association with an RNAPII containing complex under these more stringent conditions (Figure 3B, lanes 2 and 6).

To address whether the CBC interacts with the transcription machinery as a functional complex, the co-IP was carried out in strains lacking one or the other of the two CBC subunits. The CBC-RNAPII interaction is lost in strains that do not contain an intact CBC (Figure 3B, lanes 3, 4, 7, and 8). In strains deleted of *CBP20*, we find that Cbp80 alone does not detectably associate with RNAPII. Although the deletion of *CBP80* is found to decrease the stability of Cbp20 as previously reported (Fortes, Kufel et al. 1999), we are still able to pull down tagged Cbp20 and do not observe a Cbp20-RNAPII interaction in *cbp80Δ* strains.

To be certain that the IgG beads used in the TAP-immunoprecipitation do no nonspecifically bind the RNAPII, an untagged wild-type strain was included as a negative control, and no Rpb1 was pulled down. Furthermore the co-IP between RNAPII and TAP-tagged CBC is specific as a TAP-tagged form of an unrelated protein, Prc1, does not co-immunoprecipitate Rpb1 (Figure 3C, lanes 1 and 4). This data further supports the hypothesis that the CBC and RNAPII associate specifically *in vivo*.

The mammalian CBC requires both subunits for the conformational change necessary for the complex to bind the 5’ cap. Furthermore the

sequence conservation between the mammalian and yeast complexes suggest that the yeast CBC also binds the 5' cap as a heterodimer (Mazza, Ohno et al. 2001; Mazza, Segref et al. 2002). So we considered the possibility that the CBC interaction with RNAPII could be mediated through the nascent, capped RNA. This cross-link co-immunoprecipitation protocol permits us to test the RNA dependence of the CBC-RNAPII interaction by introducing an RNase treatment step prior to immunoprecipitating the tagged proteins. If RNA is the bridge between the CBC and RNAPII, this RNase treatment should abrogate the CBC-RNAPII interaction. However, we observe little change in the CBC-RNAPII interaction even with RNase treatment (Figure 3C, compare lanes 2 to 3 and 8 to 9). We confirmed that the RNase is active under these conditions by examining the RNA from crosslinked cells following treatment with 1/10th the amount of RNase used in Figure 3B (Supplementary Figure 1, lanes 3, 6, 9, 12), confirming that the conditions of the RNase step are sufficient to degrade RNA and further suggesting that the nascent RNA does not bridge the interaction we observe between the CBC and RNAPII. An interaction between RNAPII and the CBC appears to be conserved; in mammalian cells, Cbp80 has also been found to co-immunoprecipitate with RNAPII (Lejeune, Ishigaki et al. 2002), although the RNA-dependence of this interaction was not addressed.

The RNase treatment experiments suggest that the CBC is interacting with RNAPII through protein-protein interactions. Other studies have shown

that RNA processing proteins bind directly to the CTD of RNAPII, and we considered the possibility that such an interaction may exist between the CBC and the CTD. We have not been able to observe this direct interaction in preliminary experiments using a CTD peptide and immunoprecipitated CBC (data not shown). While we do not see a direct interaction between the CBC and the CTD, and we also cannot clearly rule this possibility out, it appears that the CBC interaction with the transcription machinery cannot be strictly mediated by the CTD since RNase treatment does have some affect on the CBC-RNAPII interaction. It is also possible that other proteins are involved in this physical interaction we observe between the CBC and the transcriptional machinery.

Previous studies that show a Nrd1-CID-RNAPII interaction (Steinmetz, Ng et al. 2006; Vasiljeva, Kim et al. 2008) as well as studies showing that the CBC is a component of the Nrd1 termination complex (Vasiljeva and Buratowski 2006), raise the possibility that Nrd1 may bridge the CBC-RNAPII interaction. To test this possibility, we generated TAP-tagged strains genomically deleted of *NRD1* and carrying a plasmid copy of either wild type *NRD1* or *nrd1Δ39-169*, the form lacking the CID. Both CBC subunits remain in association with Rpb1 in the absence of the Nrd1 CID, with and without RNase treatment (Figure 3D, lanes 1 through 8). The CTD-binding control, Spt5-TAP, continues to co-immunoprecipitate with RNAPII under these same conditions

(Figure 3D, lanes 9-12). Hence, neither RNA nor the Nrd1 CID appears to be tethering the CBC to Rpb1.

To address whether this RNA independent, Nrd1-CID independent, CBC-RNAPII interaction is occurring during active transcription, we conducted chromatin immunoprecipitation (ChIP) experiments. Using the TAP-tagged Cbp80 we observe that Cbp80-TAP is present throughout *PMA1* with peak recruitment in the middle of the ORF (Figure 3E). When the immunoprecipitated sample is treated with RNase the ChIP signal on *PMA1* remains, and although the ChIP signal decreases it never drops to background levels (Figure 3E), hence while the RNA is not absolutely required for the CBC-RNAPII interaction, RNA appears to play a role in stabilizing the interaction between CBC and the transcribing machinery. In similar experiments carried out with Nrd1-TAP, we found that Nrd1 recruitment to the transcriptional machinery is severely affected by RNase treatment, suggesting that during active transcription on *PMA1* the presence of RNA is required for association of Nrd1 (Supplemental Figure 2). This is similar to results by others showing an RNA dependent association of Nrd1 to other genes (Personal communications T. Villa). These results serve as both a positive control for the ability of this RNase treatment to disrupt RNA-mediated interactions and support a model whereby neither an RNA bridge nor Nrd1 is required for CBC to associate with RNAPII during active transcription. Although these experiments do not address whether the CBC-RNAPII

interaction is direct, they do further suggest that a physical interaction between these two complexes does exist during active transcription and that the nascent RNA is not sufficient to bridge these two complexes.

Our results also demonstrate that a combination of interactions contribute to Nrd1 activity, interactions with the CTD (Figure 1), interactions with RNA (Supplemental Figure 2), and interactions with the CBC (Figure 2). Below we elucidate the mechanism by which the CBC is involved in Nrd1 regulation.

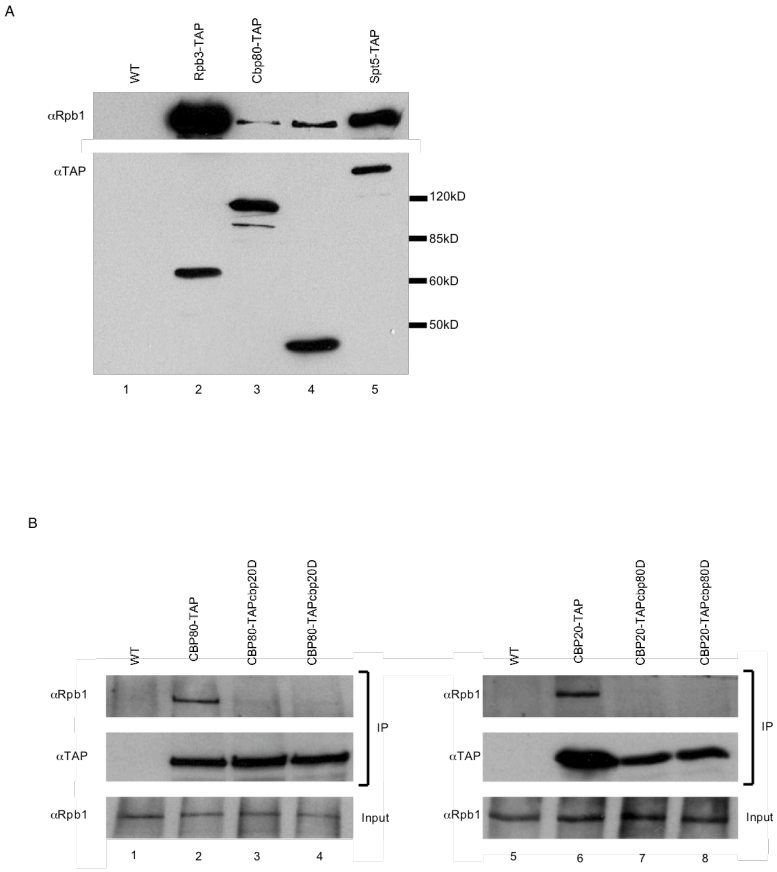


FIGURE 3. The cap-binding complex co-immunoprecipitates with the largest subunit of RNA polymerase II in an RNase-resistant, Nrd1-CID-independent manner *in vivo*. (A) Top. Immunoblot using anti-Rpb1 antibody (yN18, Santa Cruz) of the complex immunoprecipitated with the indicated TAP-tagged proteins. Bottom. Lower portion of the same blot as above but probed with anti-TAP antibody (anti-sheep IgG, HRP Upstate). Lane 1, mock immunoprecipitation using a wild type, untagged strain. Lane 2,

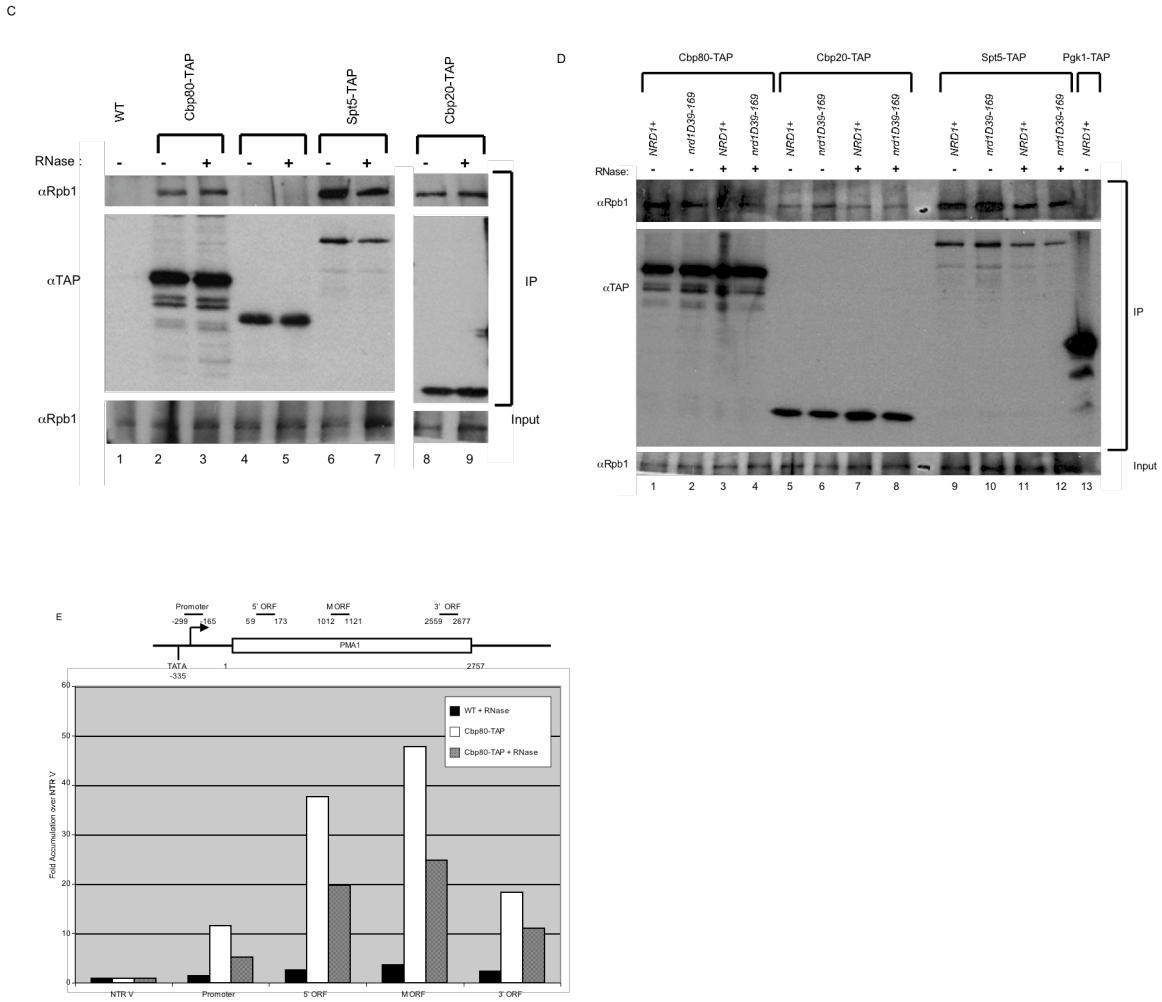


FIGURE 3 continued. co-immunoprecipitation of Rpb1 with the RNA polymerase II subunit, Rpb3. Lane 5, co-immunoprecipitation of Rpb1 with a tagged CTD-interacting protein, Spt5. A lane has been removed between lanes 4 and 5, but all of the data is from the same experiment and all the samples are shown at the same exposure for each antibody. (B) The indicated strains were crosslinked with formaldehyde prior to immunoprecipitation and

immunoblotting. The two upper panels are the co-immunoprecipitation samples run on one blot, probed for Rpb1 and the TAP-tagged protein respectively. The lower panel is the corresponding inputs probed with anti-Rpb1 antibody. (C) The indicated strains were crosslinked and treated with RNase (+) or an equal volume of sterile water (-) prior to immunoprecipitating the TAP-tagged proteins. Top and middle panels are from the same blot, cut and probed with anti-Rpb1 antibody and anti-TAP antibody, respectively. The bottom panels are the corresponding inputs probed with anti-Rpb1 antibody. Lanes 8 and 9 are from a separate blot from lanes 1-7. The same controls were run on the CBC2-TAP blot, but omitted from the figure since they are redundant. (D) The indicated strains were crosslinked and treated with RNase (+) or an equal volume of RNase buffer (-) prior to immunoprecipitating the TAP-tagged proteins. Top and middle panels are from the same blot, cut and probed separately with the indicated antibody. Pgk1 is a non-nuclear protein used as a TAP-tagged negative control. (E) ChIP of Cbp80-TAP or an untagged control (WT) in the presence or absence of RNase on *PMA1*.

CBC has functional interactions with the Rpb1-CTD of RNAPII.

Our findings of a novel CBC-RNAPII physical interaction suggest a functional relationship may also exist between the CBC and RNAPII. To address this question we employed a genetic approach to look for synthetic interactions between cells lacking the CBC subunits and harboring RNAPII mutations. We have previously observed genetic interactions between the CBC and CTD kinase complex mutants (Hossain and Johnson, submitted) suggesting this functional interaction may involve the CTD. Although complete deletion of the CTD is lethal to cells, RNAPII mutants with a truncated CTD are viable, so we examined genetic interactions between the truncated CTD and the CBC. Both *cbp80Δ* and *cbp20Δ* yeast strains have an increasingly severe growth defect as the CTD is truncated (Figure 4A and B). In fact, while truncation of the CTD to as few as 10 repeats has little effect on viability under normal growth conditions, when combined with *cbp80Δ* these cells are dead (Figure 4A). Even a truncation to 13 repeats reveals a severe synthetic growth defect. *cbp20Δ* strains are affected by CTD truncation in a similar manner (Figure 4B); when the CTD is truncated to 11 repeats or fewer repeats, cells lacking *CBP20* are nonviable. These data strongly suggest an overlap in function between the CTD repeats and the CBC. The difference seen in the genetic interactions between the two subunits of the CBC with the CTD truncations suggest that the two subunits may not have completely overlapping roles. These results are consistent with microarray expression

data indicating that the *CBP80* and *CBP20* deletions have different expression profiles (Burckin, Nagel et al. 2005).

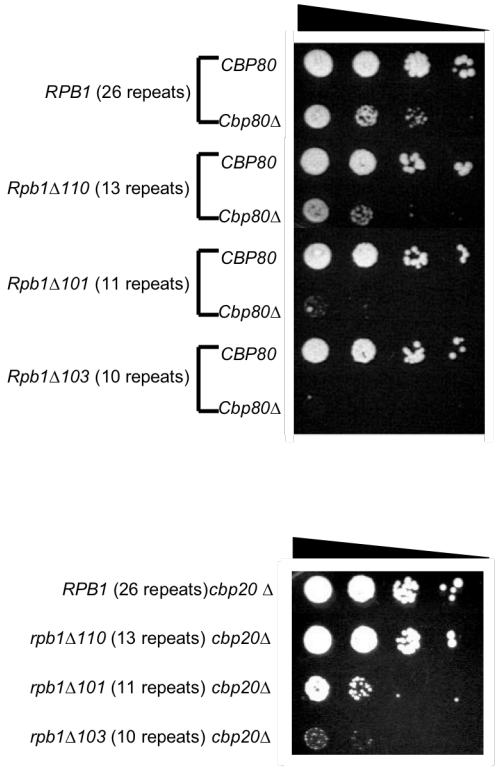


FIGURE 4. CBC has functional interactions with the CTD of the large subunit of RNAPII. (A) *cbp80Δ* shows genetic interactions with CTD truncations. *rpb1Δ* (top of each pair) or *cpb80Δrpb1Δ* (bottom of each pair) strains carrying the indicated plasmids were assayed by ten-fold serial dilutions on 5-FOA-Leu following growth in YPD to select for loss of RPB1 plasmid. The plate was incubated at 30° for 3 days prior to photographing. This set of dilution series was all grown on the same plate. (B) *cbp20Δ* shows genetic interactions with

CTD truncations. *cpb20Δrpb1Δ* strains carrying the indicated plasmids were assayed as above. Plates were incubated at 30° for 3 days prior to photographing. This set of dilution series was all grown on the same plate.

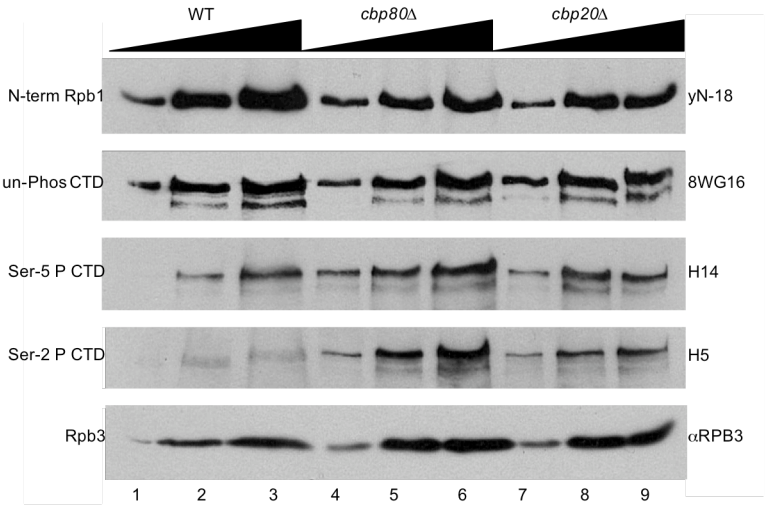


FIGURE 5. CBC mutants change the cellular CTD phosphorylation state. Western blots of total protein from WT, *cbp80Δ*, and *cbp20Δ* was probed with the indicated RNAPII antibodies. For each strain, 25μg, 75μg, and 125μg of total protein was loaded based on protein concentrations established by Bradford. The top half of each blot was probed with an anti-Rpb1 antibody and the bottom half of each blot was probed with anti-Rpb3 antibody. The anti-Rpb3 blot shown here is the bottom portion of the yN-18 blot and is representative of the anti-Rpb3 blots from this set of cellular extracts.

CBC alters the cellular CTD phosphorylation state.

These findings of a functional interaction between the RNAPII CTD and the CBC led us to examine the relationship between the CBC and CTD phosphorylation. Previous studies show that capping enzymes can alter CTD

phosphorylation and coupled to our previous findings of synthetic lethality between the CBC and CTD kinase deletions (Hossain and Johnson, submitted), we examined the possibility of such role for the CBC. We compared cellular levels of CTD phosphorylation in wild-type cells and cells deleted of the CBC subunits. Indeed deletion of the CBC subunits leads to global changes in CTD phosphorylation (Figure 5). An N-terminal specific Rpb1 antibody and an anti-Rpb3 antibody were used to control for RNAPII levels in the different strains. The unphosphorylated form of the CTD appears unaffected by deletion of the CBC subunits, and there are minor affects on the serine-5 phosphorylation. Strikingly there is a significant increase in the levels of the phosphorylated serine-2 in strains lacking either CBC subunit, suggesting that the CBC normally helps to modulate serine-2 phosphorylation levels, either by repressing the kinases involved in serine-2 phosphorylation or by stimulating the phosphatase acting on the serine-2 residue. Cellular levels of the CTK and Bur complexes are unchanged when the CBC is deleted (Hossain and Johnson, submitted).

The different phosphorylation states of the CTD have been correlated with different stages of transcription and facilitate interactions between the polymerase and both RNA processing factors and transcription factors, including Nrd1. Since CTD-binding proteins have been shown to affect CTD phosphorylation, we also looked for a change in CTD phosphorylation in the Nrd1-CID mutant and observed no change (Supplemental Figure 3).

The ratio of serine-2 to serine-5 phosphorylation is critical for Nrd1 interaction with the transcription machinery (Gudipati, Villa et al. 2008). Hence the dramatic shift in serine-2 phosphorylation, leads us to hypothesize that the CBC directs Nrd1 complex activity by facilitating proper binding of Nrd1 to the CTD. Furthermore termination of short RNA transcripts by the Nrd1 complex likely requires stabilization of Nrd1 interactions with the transcription machinery through the CTD interaction domain.

Proteins involved in CTD phosphorylation alter *IMD2* transcription attenuation.

If the CBC effect on CTD-phosphorylation underlies Nrd1-dependent termination of CUTs, we expect that deletion of components of the BUR and CTK complexes, both implicated in CTD phosphorylation, will also result in Nrd1/Sen1 termination defects for *IMD2* CUTs. By northern, we find that the same *IMD2* phenotype seen in CBC deletion mutants is also seen in the *bur2Δ*, *ctk2Δ*, and *ctk3Δ* strains under uninduced conditions (Figure 6, compare lane 2 to lanes 3-5). Although the CBC is still present in these deletion strains, changing the CTD phosphorylation state via the CTD kinases also alters Nrd1 complex function. This reinforces the importance of the correct CTD phosphorylation state for Nrd1 complex termination of the *IMD2* CUTs, but also that direct physical interactions between the CBC and the Nrd1 complex are unlikely how Nrd1 complex termination is regulated. The CBC

and the CTD kinases may be involved in redundant pathways to regulate Nrd1 complex function, but both sets of factors are clearly acting on CTD phosphorylation. The role of the CBC in regulating CTD phosphorylation is likely to have a wide range of effects on co-transcriptional processes, of which regulating Nrd1 termination is one of them. The effects of the CBC on CTD phosphorylation and its downstream consequences bear further study.

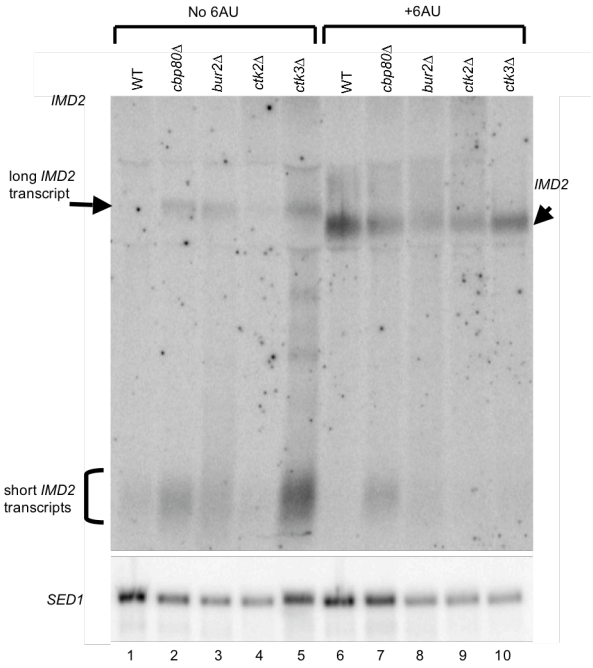


FIGURE 6. Transcription elongation factors implicated in CTD phosphorylation exhibit similar *IMD2* attenuation defects to CBC mutants. Northern blot of *IMD2* transcripts in WT and *cbp80Δ* strains for comparison to deletions of components of the BUR and CTK complexes. Bottom panel is the same blot stripped and re-probed for *SED1* as a loading control.

DISCUSSION

The CBC has been shown to act at multiple steps in RNA processing. Here we show novel activities of the CBC including interactions with RNAPII (Figure 3 and 4), modulation of the CTD phosphorylation state (Figure 5), and effects on recruitment of Nrd1 during active transcription (Figure 2), all of which are likely contributing to regulation of the Nrd1 complex dependent termination of CUTs (Figure 1), but also suggest that the CBC may be affecting other processes during transcription. The CBC has been linked to co-transcriptional recruitment of splicing factors previously (Görnemann, Kotovic et al. 2005) without any implication of CTD involvement, but our findings regarding the CBC-RNAPII interaction suggest that the CBC may also be involved in recruitment or release of many factors co-transcriptionally to or from the CTD. The CBC may be a key coordinator of RNA processing and transcription through its interactions with the CTD.

The CBC has genetic and functional interactions with RNAPII

In the experiments described above, the CBC and Rpb1 co-immunoprecipitate, in a manner that maintains *in vivo* interactions and permits us to examine whether RNA is bridging the interaction. In a previous study, the Rpb1 was used to immunoprecipitate the CBC and was found to have an interaction that was RNA dependent (Tardiff, Abruzzi et al. 2007). When we use the CBC to immunoprecipitate Rpb1, we observe RNA-independent

interactions. The difference is likely due to the abundance of Rpb1 in the cell, some of which is involved in active transcription at different stages, some of which is poised to begin transcription, a minority of which is available to co-immunoprecipitate with the CBC. The CBC, we believe, has a much larger proportion of the protein involved in protein-protein interactions with Rpb1, than the Rpb1 does with the CBC. Consistent with this, while there is some decrease in association of the CBC with the *PMA1* when the samples are treated with RNase, but the association is never reduced to background levels, as we observe when Nrd1 ChIP experiments are carried out with a RNase step. Since Nrd1 is known to bind both directly to the RNA and directly to the CTD (Supplemental Figure 2), it appears that the CBC has a much more robust interaction with the transcription machinery than Nrd1 does. This interaction appears to be evolutionarily conserved, as the mammalian CBC was initially co-immunoprecipitated with an antibody against the CTD (Lejeune, Ishigaki et al. 2002), although the mammalian studies did not address whether this was a direct protein-protein interaction, whether RNA is involved, or whether the interaction occurred during transcription. Our studies do not rule out a role for the 5' cap in the interactions we have identified between the CBC and the RNAPII. The 5' cap maybe involved in the initial association of CBC with the transcriptional machinery or may be required for the conformational changes that Cbp80 and Cbp20 undergo to bind to each

other and interact with the transcription machinery (Figure 3B) (Mazza, Ohno et al. 2001; Mazza, Segref et al. 2002).

Although we do not observe direct protein-protein interactions between the CBC and the CTD, we do see functional evidence of a relationship between these as deletion of the CBC renders cells dependent on an intact CTD (Figure 4) and fully functional CTK and Bur complexes (CTD phosphorylation complexes). Strikingly, when the CBC subunits are deleted we see a significant change in global CTD phosphorylation (Figure 5). One downstream effect of misregulation of CTD phosphorylation is defective Nrd1 complex termination activity. We expect that further examination of factors whose activities depend on CTD phosphorylation will reveal a dependence on the CBC, including factors that coordinate RNA processing with transcription.

Functional requirements for Nrd1 termination complex activity

The Nrd1 complex function is mediated by a number of interactions: Nrd1 and Nab3 need to interact with the RNA, the complex needs to interact with RNAPII, and the complex requires a Sen1/*IMD2* terminator sequence in the RNA. We propose that the CBC is involved mostly in the Nrd1 complex interactions with RNAPII. Consistent with an interaction between the Nrd1 complex and the RNA independent of CBC/RNAPII interactions, we find that RNase treatment will abolish interactions between Nrd1 and the transcriptional machinery during active transcription (Supplemental Figure 2). Intriguingly, *PMA1* is a gene locus where: Nrd1 is interacting with the RNA during active

transcription (Supplemental Figure 2), and Nab3 is likely also associated as the RNA sequence contains multiple Nab3 recognition sites; and the Nrd1 complex also appears to be interacting with the transcriptional machinery in a CBC dependent manner (Figure 2); and yet has not been shown to be terminated by the Nrd1 complex to create short transcripts as opposed to the normal full length transcript. We propose that the key difference between *IMD2* and *PMA1*, which determines whether the Nrd1 complex terminates transcription, is the lack of a Sen1/*IMD2* terminator sequence in *PMA1*.

The interaction between the Nrd1 complex and the RNA is critical for Nrd1 termination. All of the components of the Nrd1 termination complex, even with the CBC included, feature domains involved in RNA binding: Sen1 is a RNA helicase, the CBC binds to the 5' methylguanosine cap and may contain additional RNA interaction domains, and Nrd1 and Nab3 have sequence specific RNA binding domains that recognize GUAA/G or UCUU respectively (Carroll, Pradhan et al. 2004). Both of these four nucleotide sequences recognized by Nrd1 and Nab3 are quite ubiquitous in transcribed RNA, which suggests that these proteins may be found generally on RNA, including those that do not require their involvement for termination. In this study we find that even the interaction between Nrd1 and the RNAPII, although previously shown to be direct through binding of Nrd1 to the serine-5 phosphorylated form of the CTD (Vasiljeva and Buratowski 2006), can be abrogated by RNase treatment prior to co-immunoprecipitation (Supplemental Figure 2).

Tethering Nrd1 to the 5’ of a reporter RNA was sufficient to elicit termination and degradation, but not to the 3’ end (Gudipati, Villa et al. 2008), suggesting that binding to RNA is not sufficient, but something additional in the 5’ end of the RNA is required for Nrd1 complex activity separate from binding to the RNA. The CBC is typically found at the 5’ end of RNA, and should be found there in this study as well. Consistent with the CBC’s involvement in Nrd1 function at the 5’ end of RNA, we also do not find that the CBC is involved in the initial recruitment of Nrd1 to sites of transcription, as the complete loss of the CBC subunits does not lead to a general decrease in Nrd1 recruitment, but instead a very specific decrease in the 5’ end of *PMA1* is observed (Figure 2). The initial recruitment of Nrd1 to the transcription machinery is likely through binding to the nascent RNA transcript. It does not appear to us that the physical involvement of the CBC with the Nrd1 complex is required for the Nrd1 termination complex function, despite a physical association previously described between the CBC and the Nrd1 complex, rather we observe that the interactions between the CBC and the RNAPII, in particular the effects we observe on the CTD phosphorylation, are more critical to Nrd1 complex activity.

A second requirement for Nrd1 termination complex function is the phosphorylation state of the CTD. Nrd1 interacts directly with the serine-5 phosphorylated CTD through its CID, and Nrd1 activity is antagonized by serine-2 phosphorylation on the CTD. We hypothesize that the CBC affects

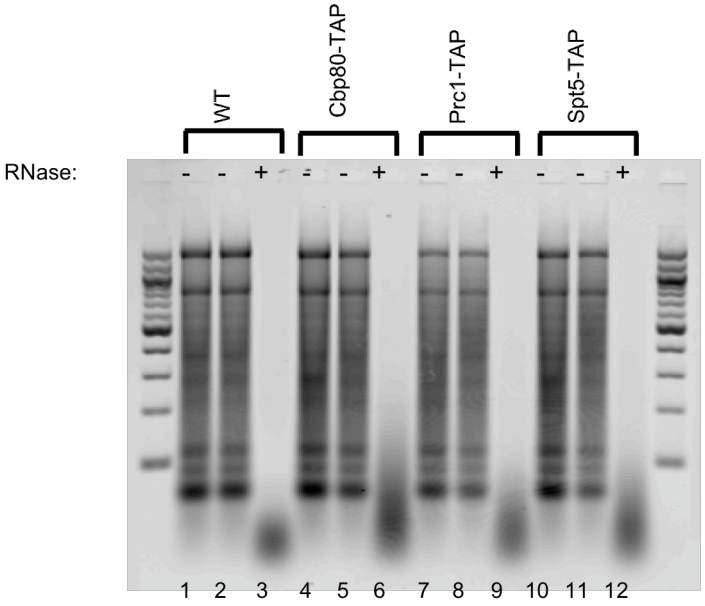
this interaction since we find that deletion of the CBC subunits lead to a global increase in serine-2 phosphorylation (Figure 5). The Nrd1 CID mutant, deletion of CTD kinase subunits, or deletion of CBC subunits all show a similar defect in *IMD2* CUTs (Figures 1A, 1B, and 6), suggesting that these factors are all involved in this process. Double mutants of the Nrd1 CID and deletion of *CBP80* do not show an exacerbated phenotype (data not shown), suggesting that the Nrd1 CID and the CBC have an overlapping function in dictating Nrd1 complex termination.

PMA1, a gene, we used to examine Nrd1 recruitment in this study meets both of the Nrd1 complex termination requirements that we have described above, yet it is not prematurely terminated by the Nrd1 complex in the 5' end. We suspect that this is because *PMA1*, unlike the *IMD2* locus, lacks a Sen1 terminator sequence. This is likely to be the final requirement for Nrd1 complex termination, but it may be possible to bypass this requirement by tethering Nrd1 tightly to the RNA.

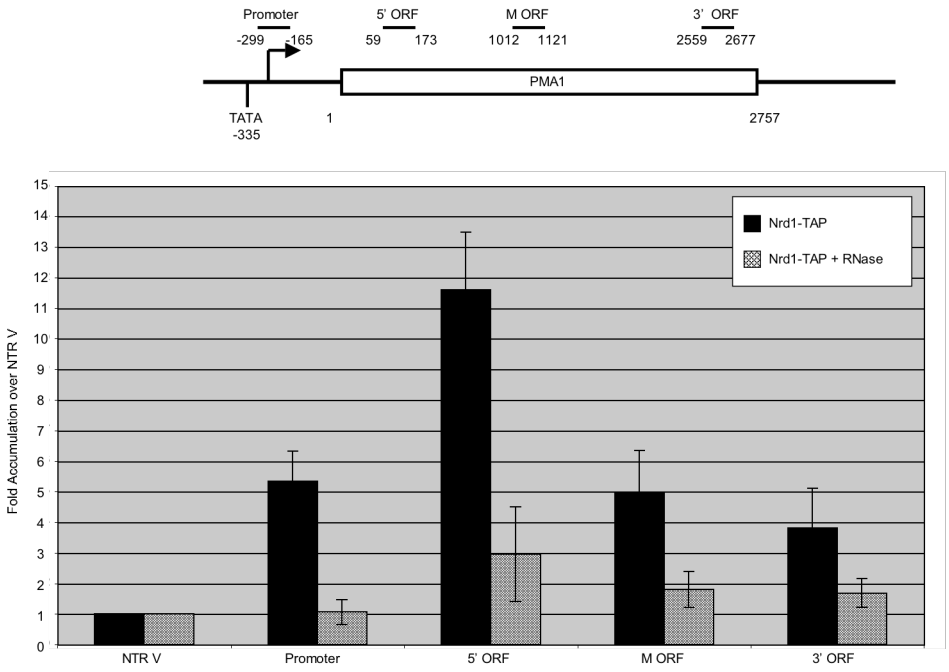
We propose that following Nrd1 and Nab3 binding to the RNA, a conformational change may be affected by interaction with RNAPII CTD through the Nrd1 CID allowing the Nrd1 complex to be more tightly associated with the RNA and once a termination sequence is detected in this region, the Nrd1 complex is able to carry out its termination activities.

Conclusion

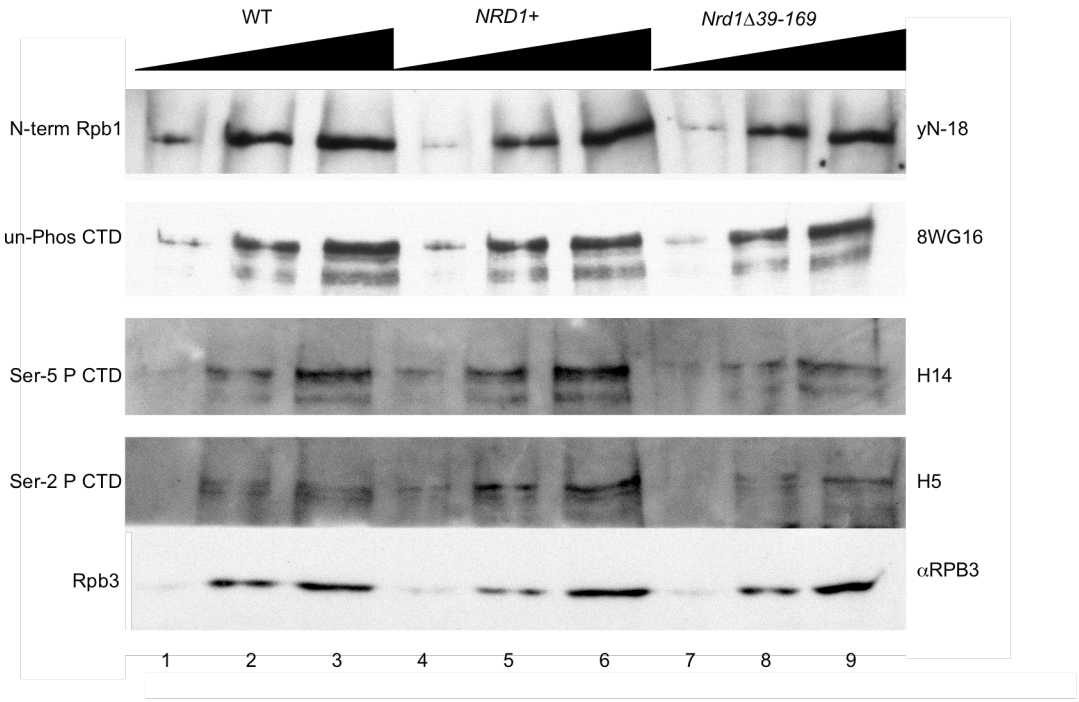
Here we find a novel role for the CBC in termination through the Nrd1 complex, which has led us to look further at interactions between the CBC and RNAPII. The CBC plays a critical role in termination of CUTs, but consistent with previous studies, this did not entail a role in transcription elongation. Furthermore, we find that the CBC, is not only associated with the Nrd1 complex, but that it is actively involved in regulating Nrd1 function. This permits us to present a model that is consistent with both our findings and the current literature for how Nrd1 complex activity may be directed to act only in designated sites of termination. These studies further elucidate novel interactions between the CBC and RNAPII, and suggest a more extensive role for the CBC in coordinating RNA processing with the transcription machinery. It also raises the likelihood that information on the progress of transcription of a nascent RNA may be fed back to the transcribing RNAPII through the CBC.



SUPPLEMENTAL FIGURE 1. Effect of RNase treatment on RNA during crosslink immunoprecipitation. Cells were treated as described in Figure 1B. Pre-cleared cell lysate was subjected to phenol chloroform extraction from 20 μL samples collected prior to incubation with CL-4B beads (lanes 1, 4, 7, 10). Samples shown in lanes 3, 6, 9, and 12 were treated with 1μg/mL RNase A; while samples in lanes 2, 5, 8, 11 were treated with an equal volume of water. Total RNA was then separated on a 2% agarose gel. The amount of RNase A used in this experiment is 10-fold less than that used in Figure 1B.



SUPPLEMENTAL FIGURE 2. Nrd1 recruitment to the transcriptional machinery is dependent on RNA. ChIP of Nrd1-TAP in the presence or absence of RNase on *PMA1*.



SUPPLEMENTAL FIGURE 3. Nrd1 CID does not affect RNAPII CTD phosphorylation. Western blots of total protein from WT, *NRD1+* (*nrd1Δ* carrying a WT copy of *NRD1* on a plasmid), and *nrd1Δ39-169* strains was probed with the indicated RNAPII antibodies. For each strain, 25μg, 75μg, and 125μg of total protein was loaded based on protein concentrations established by Bradford. The top half of each blot was probed with an anti-Rpb1 antibody and the bottom half of each blot was probed with anti-Rpb3 antibody. The anti-Rpb3 blot shown here is the bottom portion of the yN-18 blot and is representative of the anti-Rpb3 blots from this set of whole cell extracts.

MATERIALS AND METHODS

Yeast strains and methods: All strains used in these studies are derived from BY4741/BY4742 (heterozygous diploid) of the Saccharomyces Genome Deletion project (Winzeler, Shoemaker et al. 1999) and purchased from Open Biosystems. Generation of double mutants was carried out following standard procedure. For the *rpb1*Δ and *nrd1Δ* strains, heterozygous diploid cells were transformed with a WT copy of the deleted gene in a pRS316 plasmid vector prior to dissection. All yeast transformations were carried out using lithium acetate treatment as previously described and plated onto the appropriate selective media to retain the marked plasmids.

For the dilution spot assays with CTD truncated cells, strains were grown overnight at 30° in 5 mL of SC-Ura-Leu liquid media, then diluted 10-fold and grown to the same optical density at 600nm ($O.D._{600}$). The *cbp80*Δ cells were plated at $O.D._{600}$ 0.37 ±0.01 and *cbp20*Δ cells were plated at $O.D._{600}$ 0.40 ±0.01 at 30°. 10-fold serial dilutions were then made with YPD and 4μL of each dilution was plated onto 5-FOA-Leu.

Plasmids: All *RPB1* plasmids were provided courtesy of the R. Young Lab except for pRP001, the *RPB1-LEU* plasmid, generated by digesting pRP112 and the pRP114 with *Sph*1 and *Pvu*II and inserting the pRP112 insert into the pRP114 backbone. All *NRD1* plasmids have been previously described (Steinmetz and Brow 1996).

RNA analysis: The yeast strains were transformed with pRS316 and were grown in SC-URA media to $O.D._{600}$ of about 0.5 at 30° and divided into two aliquots. 6AU was added to one half of the culture to a final concentration of 100 µg/mL and the cultures were returned to 30°. Cells were then harvested after 2 hours, with 0 hour being the time of 6AU addition, and flash frozen in liquid nitrogen. Total RNA was isolated from thawed cells using a hot phenol treatment and quantitated by UV spectrophotometer. An equal amount of total RNA from each strain was resolved on a 1.5% agarose-formaldehyde gel and then transferred to Zeta-probe membrane (Bio-Rad) by capillary transfer. *IMD2* probe was generated by PCR using primers previously described (Davis and Ares 2006). Each DNA fragment was radiolabeled using [α-^{32}P] dCTP with the Rediprime Kit (Amersham Biosciences) and used for hybridization. The images were captured using a Typhoon phosphorImager (GE Healthcare) and quantitated using ImageQuant 5.2 software (GE Healthcare).

Chromatin immunoprecipitation (ChIP): The ChIP experiments were carried out as described previously (Hecht and Grunstein 1999; Zenklusen, Vinciguerra et al. 2002; Kotovic, Lockshon et al. 2003) with *PMA1* primers described previously (Zenklusen, Vinciguerra et al. 2002). For RNase A treatment, 10µg/mL of RNase A (Ambion) or equal volume of water was added to each sample at the beginning of the pre-clearing incubation.

Co-immunoprecipitation: Co-immunoprecipitation (co-IP) experiments were carried out according to the general procedure described (Wu, Wilcox et al. 2000), with the following modifications. Yeast strains were grown in 75 mL of YPD to an $O.D._{600}$ of approximately 0.8. The cells were harvested by centrifugation, washed, and lysed by vortexing with glass beads in cold lysis buffer (20mM HEPES pH 7.4; 20mM KCl; 0.5mM EDTA; pH 8.0, 0.2% Triton X-100; 1mM PMSF; 1μg/mL pepstatin; 2mM benzamidine; 2.5μg/mL leupeptin; 5μg/mL aprotinin; 200mM sodium fluoride; and 1mM activated sodium vanadate). Whole cell extracts were cleared by centrifugation in an IEC clinical centrifuge at top speed for 10 min at 4°. Cleared whole cell extracts were then incubated for 2 hr at 4° with IgG Sepharose 6 Fast Flow beads (GE Healthcare). Beads were washed with cold lysis buffer before eluting bound protein by boiling the beads for 10 min in 20μL of SDS-PAGE sample buffer. Then samples were resolved by 7.5% SDS-PAGE and transferred to nitrocellulose membrane. The membranes were cut in two, and the bottom portion of the blots were analyzed with anti-sheep IgG antibody (Upstate) to recognize the TAP-tag, while RNAPII in the top portion of the blots was detected using the with yN-18 antibody (Santa Cruz Biotechnology). Bands were detected using West Pico SuperSignal (Pierce Biotechnology) and also using ECL+ (GE Healthcare). The blots were quantified using ImageQuant 5.2 software (GE Healthcare).

Crosslink-immunoprecipitation (XIP): Strains were grown in 150mL of YPD to an $O.D._{600}$ of approximately 0.5 and crosslinked in 1% formaldehyde for 15 min, followed by a 5 minute incubation with glycine at a final concentration of 130mM. The pellets were washed in 1X PBS, resuspended in FA-1 lysis buffer containing protease inhibitors (50mM HEPES-KOH pH7.5, 140mM NaCl, 1mM EDTA, 1% Triton-X 100, 0.1% sodium deoxycholate, 1mM PMSF, 1μg/mL Pepstatin A, and 1μg/mL Leupeptin), followed by cell lysis and sonication. To pre-clear each sample, 700μL of lysate was nutated for 1 hr at 4° with 100μL of pre-washed Sepharose CL-4B beads (Sigma). 20μL of the lysate was set aside for use as input, and the remainder was added to 50μL of pre-washed IgG Sepharose 6 Fast Flow beads (GE Healthcare). After a 2 hr incubation on the nutator at 4° with the IgG Sepharose beads, the beads were washed with cold FA-1 lysis buffer, FA-2 buffer (50mM HEPES-KOH pH7.5, 500mM NaCl, 1mM EDTA, 1% Triton-X 100, 0.1% sodium deoxycholate), FA-3 buffer (20mM Tris pH 8, 250mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1mM EDTA), and finally with TE. To reverse the crosslinks 100μL of 1% SDS in TE was added to the washed beads or 80μL of 1% SDS in TE to the input, and the samples were incubated overnight at 65°. SDS sample buffer was added to the XIP samples and inputs and boiled for 5 minutes prior to loading 20μL of XIP mix or 10μL of input mix on a 7.5% SDS-PAGE. Immunoblotting was performed as described above. For RNase A

treatment, 10μg/mL of RNase A (Ambion) or equal volume of water was added to each sample at the beginning of the Sepharose CL-4B bead incubation.

CTD-Phosphorylation Immunoblots: Yeast cells were grown to an $O.D._{600}$ of 0.8., washed, and lysed by vortexing with glass beads in the same cold lysis buffer described previously for co-immunoprecipitation with the addition of 10% glycerol. Whole cell extracts were cleared by centrifugation in an IEC clinical centrifuge at top speed for 10 minutes at 4°. Total protein from this whole cell extract was quantitated by Bradford protein assay, and loaded in a gradient of 25μg, 75μg, and 125μg onto a 7.5% SDS-PAGE. For immunoblotting, the membranes are cut in two and the top portion was probed for Rpb1 using either yN-18 (N-terminus, Santa Cruz Biotechnology, Inc.), 8WG16, H5, or H14 (Covance). The bottom portion was probed for Rpb3 (anti-Rpb3, NeoClone Biotechnology) to verify equal loading.

Chapter 2, in part is currently being prepared for submission for publication with T.L. Johnson as the corresponding author with the working title “The *S. cerevisiae* Nuclear Cap Binding Complex Interacts with RNA polymerase II to Regulate Nrd1 Complex Function." The dissertation author was the primary author on this manuscript.

REFERENCES

Aguilera, A. (2005). "Cotranscriptional mRNP assembly: from the DNA to the nuclear pore." Curr Opin Cell Biol 17(3): 242-50.

Burckin, T., R. Nagel, et al. (2005). "Exploring functional relationships between components of the gene expression machinery." Nat Struct Mol Biol 12(2): 175-82.

Carroll, K. L., D. A. Pradhan, et al. (2004). "Identification of cis elements directing termination of yeast nonpolyadenylated snoRNA transcripts." Mol Cell Biol 24(14): 6241-52.

Cho, E. J., M. S. Kobor, et al. (2001). "Opposing effects of Ctk1 kinase and Fcp1 phosphatase at Ser 2 of the RNA polymerase II C-terminal domain." Genes Dev 15(24): 3319-29.

Cho, E. J., T. Takagi, et al. (1997). "mRNA capping enzyme is recruited to the transcription complex by phosphorylation of the RNA polymerase II carboxy-terminal domain." Genes Dev 11(24): 3319-26.

Clifton, D. and D. G. Fraenkel (1981). "The gcr (glycolysis regulation) mutation of Saccharomyces cerevisiae." J Biol Chem 256(24): 13074-8.

Clifton, D., S. B. Weinstock, et al. (1978). "Glycolysis mutants in Saccharomyces cerevisiae." Genetics 88(1): 1-11.

Corden, J. L. and M. Patturajan (1997). "A CTD function linking transcription to splicing." Trends Biochem Sci 22(11): 413-6.

Culbertson, M. R. and E. Neeno-Eckwall (2005). "Transcript selection and the recruitment of mRNA decay factors for NMD in Saccharomyces cerevisiae." RNA 11(9): 1333-9.

Das, B., J. S. Butler, et al. (2003). "Degradation of normal mRNA in the nucleus of Saccharomyces cerevisiae." Mol Cell Biol 23(16): 5502-15.

Das, B., S. Das, et al. (2006). "Mutant LYS2 mRNAs retained and degraded in the nucleus of Saccharomyces cerevisiae." Proc Natl Acad Sci U S A 103(29): 10871-6.

Davis, C. A. and M. Ares, Jr. (2006). "Accumulation of unstable promoter-

associated transcripts upon loss of the nuclear exosome subunit Rrp6p in Saccharomyces cerevisiae." Proc Natl Acad Sci U S A 103(9): 3262-7.

Du, H. and M. Rosbash (2001). "Yeast U1 snRNP-pre-mRNA complex formation without U1snRNA-pre-mRNA base pairing." Rna 7(1): 133-42.

Fan, H. Y., K. K. Cheng, et al. (1996). "Mutations in the RNA polymerase II transcription machinery suppress the hyperrecombination mutant hpr1 delta of Saccharomyces cerevisiae." Genetics 142(3): 749-59.

Fan, H. Y. and H. L. Klein (1994). "Characterization of mutations that suppress the temperature-sensitive growth of the hpr1 delta mutant of Saccharomyces cerevisiae." Genetics 137(4): 945-56.

Flaherty, S. M., P. Fortes, et al. (1997). "Participation of the nuclear cap binding complex in pre-mRNA 3' processing." Proc Natl Acad Sci U S A 94(22): 11893-8.

Fortes, P., D. Bilbao-Cortes, et al. (1999). "Luc7p, a novel yeast U1 snRNP protein with a role in 5' splice site recognition." Genes Dev 13(18): 2425-38.

Fortes, P., J. Kufel, et al. (1999). "Genetic and physical interactions involving the yeast nuclear cap-binding complex." Mol Cell Biol 19(10): 6543-53.

Gornemann, J., K. M. Kotovic, et al. (2005). "Cotranscriptional spliceosome assembly occurs in a stepwise fashion and requires the cap binding complex." Mol Cell 19(1): 53-63.

Gudipati, R. K., T. Villa, et al. (2008). "Phosphorylation of the RNA polymerase II C-terminal domain dictates transcription termination choice." Nat Struct Mol Biol 15(8): 786-94.

Hecht, A. and M. Grunstein (1999). "Mapping DNA interaction sites of chromosomal proteins using immunoprecipitation and polymerase chain reaction." Methods Enzymol 304: 399-414.

Holland, M. J., T. Yokoi, et al. (1987). "The GCR1 gene encodes a positive transcriptional regulator of the enolase and glyceraldehyde-3-phosphate dehydrogenase gene families in Saccharomyces cerevisiae." Mol Cell Biol 7(2): 813-20.

Hosoda, N., Y. K. Kim, et al. (2005). "CBP80 promotes interaction of Upf1

with Upf2 during nonsense-mediated mRNAmammalian cells." Nat Struct Mol Biol 12(10): 893-901.

Jenks, M. H., T. W. O'Rourke, et al. (2008). "Properties of an intergenic terminator and start site switch that regulate IMD2 transcription in yeast." Mol Cell Biol 28(12): 3883-93.

Kawasaki, G. and D. G. Fraenkel (1982). "Cloning of yeast glycolysis genes by complementation." Biochem Biophys Res Commun 108(3): 1107-22.

Keogh, M. C., V. Podolny, et al. (2003). "Bur1 kinase is required for efficient transcription elongation by RNA polymerase II." Mol Cell Biol 23(19): 7005-18.

Kopcewicz, K. A., T. W. O'Rourke, et al. (2007). "Metabolic regulation of IMD2 transcription and an unusual DNA element that generates short transcripts." Mol Cell Biol 27(8): 2821-9.

Kotovic, K. M., D. Lockshon, et al. (2003). "Cotranscriptional recruitment of the U1 snRNP to intron-containing genes in yeast." Mol Cell Biol 23(16): 5768-79.

Lejeune, F., Y. Ishigaki, et al. (2002). "The exon junction complex is detected on CBP80-bound but not eIF4E-bound mRNA in mammalian cells: dynamics of mRNP remodeling." Embo J 21(13): 3536-45.

Lewis, J. D., D. Gorlich, et al. (1996). "A yeast cap binding protein complex (yCBC) acts at an early step in pre-mRNA splicing." Nucleic Acids Res 24(17): 3332-6.

Lewis, J. D. and E. Izaurralde (1997). "The role of the cap structure in RNA processing and nuclear export." Eur J Biochem 247(2): 461-9.

Lindstrom, D. L. and G. A. Hartzog (2001). "Genetic interactions of Spt4-Spt5 and TFIIS with the RNA polymerase II CTD and CTD modifying enzymes in Saccharomyces cerevisiae." Genetics 159(2): 487-97.

Maniatis, T. and R. Reed (2002). "An extensive network of coupling among gene expression machines." Nature 416(6880): 499-506.

Mazza, C., M. Ohno, et al. (2001). "Crystal structure of the human nuclear cap binding complex." Mol Cell 8(2): 383-96.

Mazza, C., A. Segref, et al. (2002). "Co-crystallization of the human nuclear cap-binding complex with a m7GpppG cap analogue using protein engineering." Acta Crystallogr D Biol Crystallogr 58(Pt 12): 2194-7.

McCracken, S., N. Fong, et al. (1997). "5'-Capping enzymes are targeted to pre-mRNA by binding to the phosphorylated carboxy-terminal domain of RNA polymerase II." Genes Dev 11(24): 3306-18.

Mili, S. and J. A. Steitz (2004). "Evidence for reassociation of RNA-binding proteins after cell lysis: implications for the interpretation of immunoprecipitation analyses." Rna 10(11): 1692-4.

Morris, D. P. and A. L. Greenleaf (2000). "The splicing factor, Prp40, binds the phosphorylated carboxyl-terminal domain of RNA polymerase II." J Biol Chem 275(51): 39935-43.

Murray, S., R. Udupa, et al. (2001). "Phosphorylation of the RNA polymerase II carboxy-terminal domain by the Bur1 cyclin-dependent kinase." Mol Cell Biol 21(13): 4089-96.

Narita, T., T. M. Yung, et al. (2007). "NELF interacts with CBC and participates in 3' end processing of replication-dependent histone mRNAs." Mol Cell 26(3): 349-65.

O'Mullane, L. and I. C. Eperon (1998). "The pre-mRNA 5' cap determines whether U6 small nuclear RNA succeeds U1 small nuclear ribonucleoprotein particle at 5' splice sites." Mol Cell Biol 18(12): 7510-20.

Palancade, B. and O. Bensaude (2003). "Investigating RNA polymerase II carboxyl-terminal domain (CTD) phosphorylation." Eur J Biochem 270(19): 3859-70.

Phatnani, H. P. and A. L. Greenleaf (2006). "Phosphorylation and functions of the RNA polymerase II CTD." Genes Dev 20(21): 2922-36.

Rasmussen, T. P. and M. R. Culbertson (1998). "The putative nucleic acid helicase Sen1p is required for formation and stability of termini and for maximal rates of synthesis and levels of accumulation of small nucleolar RNAs in Saccharomyces cerevisiae." Mol Cell Biol 18(12): 6885-96.

Riles, L., R. J. Shaw, et al. (2004). "Large-scale screening of yeast mutants for sensitivity to the IMP dehydrogenase inhibitor 6-azauracil." Yeast 21(3): 241-8.

Santos-Rosa, H. and A. Aguilera (1995). "Isolation and genetic analysis of

extragenic suppressors of the hyper-deletion phenotype of the Saccharomyces cerevisiae hpr1 delta mutation." Genetics 139(1): 57-66.

Steinmetz, E. J. and D. A. Brow (1996). "Repression of gene expression by an exogenous sequence element acting in concert with a heterogeneous nuclear ribonucleoprotein-like protein, Nrd1, and the putative helicase Sen1." Mol Cell Biol 16(12): 6993-7003.

Steinmetz, E. J., N. K. Conrad, et al. (2001). "RNA-binding protein Nrd1 directs poly(A)-independent 3'-end formation of RNA polymerase II transcripts." Nature 413(6853): 327-31.

Steinmetz, E. J., S. B. Ng, et al. (2006). "cis- and trans-Acting determinants of transcription termination by yeast RNA polymerase II." Mol Cell Biol 26(7): 2688-96.

Tardiff, D. F., K. C. Abruzzi, et al. (2007). "Protein characterization of Saccharomyces cerevisiae RNA polymerase II after in vivo cross-linking." Proc Natl Acad Sci U S A 104(50): 19948-53.

Uemura, H. and Y. Jigami (1992). "GCR3 encodes an acidic protein that is required for expression of glycolytic genes in Saccharomyces cerevisiae." J Bacteriol 174(17): 5526-32.

Uemura, H., S. Pandit, et al. (1996). "Mutations in GCR3, a gene involved in the expression of glycolytic genes in Saccharomyces cerevisiae, suppress the temperature-sensitive growth of hpr1 mutants." Genetics 142(4): 1095-103.

Ursic, D., K. L. Himmel, et al. (1997). "The yeast SEN1 gene is required for the processing of diverse RNA classes." Nucleic Acids Res 25(23): 4778-85.

Vasiljeva, L. and S. Buratowski (2006). "Nrd1 interacts with the nuclear exosome for 3' processing of RNA polymerase II transcripts." Mol Cell 21(2): 239-48.

Vasiljeva, L., M. Kim, et al. (2008). "The Nrd1-Nab3-Sen1 termination complex interacts with the Ser5-phosphorylated RNA polymerase II C-terminal domain." Nat Struct Mol Biol 15(8): 795-804.

Winzeler, E. A., D. D. Shoemaker, et al. (1999). "Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis." Science 285(5429): 901-6.

Wong, C. M., H. Qiu, et al. (2007). "Yeast cap binding complex impedes recruitment of cleavage factor IA to weak termination sites." Mol Cell Biol 27(18): 6520-31.

Wu, X., C. B. Wilcox, et al. (2000). "The Ess1 prolyl isomerase is linked to chromatin remodeling complexes and the general transcription machinery." Embo J 19(14): 3727-38.

Wyers, F., M. Rougemaille, et al. (2005). "Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase." Cell 121(5): 725-37.

Yue, Z., E. Maldonado, et al. (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 U S A 94(24): 12898-903.

Yuryev, A., M. Patturajan, et al. (1996). "The C-terminal domain of the largest subunit of RNA polymerase II interacts with a novel set of serine/arginine-rich proteins." Proc Natl Acad Sci U S A 93(14): 6975-80.

Zenklusen, D., P. Vinciguerra, et al. (2002). "Stable mRNP formation and export require cotranscriptional recruitment of the mRNA export factors Yra1p and Sub2p by Hpr1p." Mol Cell Biol 22(23): 8241-53.