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Phosphorylation of the Pol II CTD by KIN28 enhances BUR1/BUR2 recruitment and Ser2 CTD phosphorylation near promoters

Hongfang Qiu, **Cuihua Hu**, and **Alan G. Hinnebusch***
Laboratory of Gene Regulation and Development, National Institute of Child Health and Human Development, Bethesda, MD 20892

Abstract

Cyclin-dependent kinase BUR1/BUR2 appears to be the yeast ortholog of P-TEFb, which phosphorylates Ser2 of the RNA Pol II CTD, but the importance of BUR1/BUR2 in CTD phosphorylation is unclear. We show that BUR1/BUR2 is co-transcriptionally recruited to the 5′ end of *ARG1* in a manner stimulated by interaction of the BUR1 C-terminus with CTD repeats phosphorylated on Ser5 by KIN28. Impairing BUR1/BUR2 function, or removing the CTD-interaction domain in BUR1, reduces Ser2 phosphorylation in bulk Pol II and eliminates the residual Ser2P in cells lacking the major Ser2 CTD kinase, CTK1. Impairing BUR1/BUR2 or CTK1 evokes a similar reduction of Ser2P in Pol II phosphorylated on Ser5, and in elongating Pol II near the *ARG1* promoter. By contrast, CTK1 is responsible for the bulk of Ser2P in total Pol II and at promoter-distal sites. In addition to phosphorylating Ser2 near promoters, BUR1/BUR2 also stimulates Ser2P formation by CTK1 during transcription elongation.

Keywords

BUR1; BUR2; KIN28; RNA Polymerase; CTD; transcription elongation; histone methylation

The C-terminal repeat domain (CTD) of the largest subunit of RNA Pol II (RPB1) is a flexible scaffold for recruiting factors of mRNA processing, transcription elongation, or termination, whose binding is enhanced by phosphorylation of the CTD. The CTD is comprised of tandem repeats of the heptad $Y_1S_2P_3T_4S_5P_6S_7$, phosphorylated on Ser2 and Ser5 during promoter clearance and elongation (Phatnani and Greenleaf, 2006). Ser5 CTD phosphorylation (Ser5P) in vivo is catalyzed by the cyclin-dependent kinase in TFIIH (CDK7; yeast KIN28), and Ser5P is most abundant near the 5′ ends of genes (Komarnitsky et al., 2000; Schroeder et al., 2000). Ser5P promotes recruitment of mRNA capping enzyme (Cho et al., 1997; Ho and Shuman, 1999) and nuclear cap-binding complex (CBC) (Wong et al., 2007) to nascent transcripts, and co-transcriptional recruitment of elongation factor Paf1C (Qiu et al., 2006), the histone H3-Lys4 methyltransferase complex (SET1/COMPASS) (Ng et al., 2003b), and histone acetyltransferase complex SAGA (Govind et al., 2007).

Subsequent to TFIIH function, the CTD is phosphorylated on Ser2 by CDK9/P-TEFb in mammals and the CTDK-I complex in yeast (containing CTK1as catalytic subunit) (Lee and

*Corresponding author: NIH, Building 6A, Room B1A-13, Bethesda, MD 20892, Tel: (301)496-4480; Fax: (301)496-6828; email: E-mail: ahinnebusch@nih.gov.

Greenleaf, 1989; Marshall et al., 1996), and Ser2P is most abundant near the 3′ ends of genes (Cho et al., 2001; Komarnitsky et al., 2000). It is thought that P-TEFb releases elongating Pol II from pause sites induced by negative elongation factor complex NELF in concert with elongation factor DSIF (Peterlin and Price, 2006). Budding yeast lacks NELF, but it was shown that CTK1 stimulates co-transcriptional recruitment of SET2, for trimethylation of histone H3 on Lys36 (H3-K36Me3) (Krogan et al., 2003b; Li et al., 2003; Li et al., 2002; Xiao et al., 2003). CTK1 also supports 3′ end formation by enhancing recruitment of cleavage/polyadenylation factors (Ahn et al., 2004; Licatalosi et al., 2002).

BUR1 and BUR2 are the catalytic and regulatory subunits of an essential CDK in budding yeast implicated in transcription elongation (Keogh et al., 2003; Yao et al., 2000). BUR1/BUR2 are most related in sequence to P-TEFb, and it was proposed that P-TEFb's functions are divided between CTK1 and BUR1 (Keogh et al., 2003; Wood and Shilatifard, 2006). However, evidence that BUR1/BUR2 contributes directly to Ser2 phosphorylation in vivo is lacking. Consistent with the notion that BUR1/BUR2 is a Ser2 CTD kinase, *bur1* mutants are synthetically lethal with CTD truncations or *ctk1Δ*, but not with *kin28* Ts^- alleles, and *ctk1*Δ cells exhibit a weak Bur^- phenotype (suppression of *SUC2* UAS deletion). However, *kin28-ts16 bur1-2* mutants are also barely viable (Lindstrom and Hartzog, 2001), and BUR1/BUR2 phosphorylated RPB1 in immune complexes only on Ser5 (Murray et al., 2001). Using recombinant CTD substrates, BUR1 phosphorylated Ser2 and Ser5, with some preference for Ser5, and was less active than CTK1 or KIN28. Chromatin immunoprecipitation (ChIP) of RPB1 associated with the *PMA1* gene revealed no significant decreases in Ser5P or Ser2P in the catalytically defective *bur1-23* mutant. Moreover, no decrease in bulk Ser5P, and only a slight decrease in Ser2P, were detected in *bur1-23* extracts, and the Ser2P reduction was attributed to decreased amounts of elongating Pol II. Thus, BUR1's essential function seemed to involve phosphorylation of a substrate besides RPB1 (Keogh et al., 2003). On the other hand, it was reported that Ser2P is reduced only 50% by *ctk1Δ* during logarithmic growth, and CTK1 is responsible for the bulk of Ser2P only during diauxic growth (Patturajan et al., 1999). Thus, another Ser2 CTD kinase besides CTK1 must be active in exponentially growing cells.

BUR1 resembles P-TEFb in stimulating transcription elongation, but the mechanisms involved are unclear. *bur1* mutations interact with defects in various elongation factors (Chu et al., 2007; Murray et al., 2001), and resemble other elongation factor mutations in conferring sensitivity to 6-azauracil (Keogh et al., 2003; Murray et al., 2001). BUR1 and BUR2 are found throughout coding sequences, and *bur1-23* reduces Pol II occupancy in a manner indicating reduced processivity (Keogh et al., 2003). The finding that deleting subunits of histone deacetylase complex Rpd3-S suppresses the growth defects of *bur1Δ* and *bur2Δ* mutants (Keogh et al., 2005) suggests that histone hyperacetylation reduces the need for BUR1/BUR2 in elongation, perhaps by creating a less compact chromatin structure.

Another function of BUR1/BUR2 is to promote H2B monoubiquitination on Lys-123 (H2B-Ub) by RAD6/BRE1 and H3-K4 trimethylation by the SET1 complex (Laribee et al., 2005; Wood et al., 2005). Mutations in Paf1C decrease these modifications (Krogan et al., 2003a; Ng et al., 2003a; Wood et al., 2003), and considering that *bur2Δ* reduces Paf1C recruitment (Laribee et al., 2005; Qiu et al., 2006; Wood et al., 2005), the decrease in H2B-Ub and H3-K4Me3 in *bur2Δ* cells could be secondary to reduced Paf1C recruitment. There is also evidence that BUR1/BUR2 directly promotes H2B-Ub by phosphorylating RAD6 (Wood et al., 2005), but since *bre1Δ* cells lack both H2B-Ub and H3-K4Me3 and are viable, BUR1/BUR2 must have other important substrates besides RAD6. Interestingly, BUR2 also promotes SET2 recruitment and attendant H3-K36Me3 formation, particularly at the 5′ ends of genes (Chu et al., 2007).

We found previously that co-transcriptional recruitment of Paf1C by transcription factor GCN4 requires BUR2 in addition to elongation factor SPT4 and Ser5 CTD phosphorylation by KIN28 (Qiu et al., 2006). We began this study by investigating the mechanism of BUR1/BUR2 recruitment and found evidence that BUR2 recruitment to the *ARG1* gene is enhanced by KIN28. Pursuing this, we identified a CTD-interaction domain (CID) dependent on Ser5P located in the C-terminal region of BUR1, which stimulates its recruitment to the 5′ end of *ARG1*. Given that BUR2 promotes H3-K36Me3 formation at 5′ ends (Chu et al., 2007), and that SET2 recruitment is stimulated by Ser2P generated by CTK1, we asked whether BUR1/BUR2 contributes to Ser2P formation near promoters. Studying a *bur1* allele that allows chemical inhibition of kinase activity, we show that BUR1/BUR2 produces the residual Ser2P remaining when CTK1 is inactivated. We also found that BUR1/BUR2 contributes a significant proportion of Ser2P in elongating Pol II molecules phosphorylated on Ser5 and located near the promoter. We propose that BUR1/BUR2 is recruited to CTD repeats phosphorylated on Ser5 to augment Ser2 phosphorylation by CTK1 early in the transcription cycle.

Results

Co-transcriptional recruitment of BUR1/BUR2 is enhanced by KIN28

To study the mechanism of BUR1/BUR2 recruitment by GCN4, we conducted ChIP analysis of myc-tagged BUR2 binding at the *ARG1* gene in WT and *gcn4Δ* strains during isoleucine-valine starvation (imposed with the inhibitor sulfometuron) when GCN4 is induced. We normalized BUR2-myc binding at *ARG1* for non-specific binding to intergenic sequences on chromosome V (Komarnitsky et al., 2000), and for that seen in *gcn4Δ* cells, to calculate GCN4-dependent BUR2 occupancy at *ARG1*. There is strong *GCN4*-dependent recruitment of BUR2 to the *ARG1* promoter (TATA region), sequences at the 5′ or 3′ end of the coding sequence (5′ and 3′ ORF, respectively), and transcribed sequences located downstream of the ORF (Down) (Fig. 1B, WT vs. *gcn4Δ*; Fig. 1C, WT). BUR2 occupancy is generally higher in the ORF versus TATA or downstream sequences, and moderately higher at the 5′ versus 3′ end of the ORF (Fig. 1C & 1F). Moderate enrichment of BUR1-myc at the 5′ end of *GAL1* also was observed during induction by galactose (Supplementary Fig. S1). As this 5′ bias was not observed at *ARG1* for SPT5 (Fig. 1H) or CTK1 (Fig. 4F), it suggested that BUR1/BUR2 is recruited early in the elongation phase.

We have shown that deleting the *ARG1* TATA element (*ΔTATA*) reduces occupancies of SPT4, Paf1C and SAGA, in addition to Pol II itself, in *ARG1* coding sequences, indicating that their recruitment to the ORF requires transcription (Govind et al., 2007; Qiu et al., 2006). Likewise, we found that deleting TATA reduces BUR2 occupancies across *ARG1* (Fig. 1B-C, WT vs. *ΔTATA*). We then asked whether inactivating Ser5 kinase KIN28 reduces BUR2 association with transcribed *ARG1* sequences. As expected, in WT cells, Ser5P occupancy is higher in the promoter and 5′ORF than in the 3′ORF, and Ser5P is reduced in *kin28-ts16* cells at the restrictive temperature (Fig. 1D). This last effect is associated with a moderate decline in RPB3 occupancy at the 3′ORF, but not in the promoter or 5′ORF (Fig. 1E), consistent with decreased promoter clearance by Pol II (Sims et al., 2004). Importantly, BUR2 occupancy is reduced more than that of RPB3 (Fig. 1F), and the BUR2:RPB3 ratio (panel G) is reduced at all locations in *kin28-ts16* cells. These findings suggest that co-transcriptional recruitment of BUR1/BUR2 is stimulated by Ser5 phosphorylation by KIN28.

ChIP analysis of the *rtf1Δ* and *cdc73Δ* mutants, lacking Paf1C subunits necessary for Paf1C recruitment (Qiu et al., 2006), revealed no decrease in BUR2 occupancy (Fig. 1C). Similar results were obtained for an *spt4Δ* mutant (Fig. 1C) that exhibits reduced Paf1C recruitment (Qiu et al., 2006). Thus, BUR1/BUR2 recruitment is independent of Paf1C and SPT4. ChIP analysis reveals that BUR2 is dispensable for recruitment of SPT5 (Fig. 1H), and we showed previously that SPT4 recruitment was unaffected by *kin28-ts16* (Qiu et al., 2006). Together,

these results suggest that SPT4/SPT5 and BUR1/BUR2 are recruited independently by elongating Pol II, and thus make separate contributions to Paf1C recruitment, and that BUR1/BUR2 recruitment is stimulated by Ser5P.

The C-terminal region of BUR1 binds Ser5P and promotes BUR1/BUR2 recruitment

We asked next whether BUR1 or BUR2 contains a CID that could account for the stimulatory effect of KIN28 on BUR2 recruitment. Indeed, the C-terminal half of BUR1 fused to GST binds to a synthetic peptide of four CTD repeats phosphorylated on Ser5 (Ser5P-CTD), but no binding above background to the cognate unphosphorylated peptide (Fig. 2A). The N-terminal half of BUR1 and full-length BUR2 showed no binding to either peptide (Fig. 2A). By testing additional truncations of BUR1, we identified residues 351-552 as the minimal region harboring a functional CID (Fig. 2B-C). We repeated the assays with BUR1(351-602) after treating the peptide with calf intestine phosphatase (CIP) in the presence or absence of phosphatase inhibitor PhosSTOP. Treatment with CIP alone, but not in the presence of PhosSTOP, eliminated binding of BUR1(351-602) to Ser5P peptides (Fig. 2D, cf. lanes 6-7 vs. 8-9). We observed no binding to a synthetic peptide phosphorylated on Ser2 (Fig. 2D). Hence, the BUR1 CID binds to CTD repeats in a manner stimulated by Ser5 phosphorylation.

To pinpoint BUR1 residues involved in CTD binding, we identified amino acid positions highly conserved among BUR1 orthologs from different fungi, and made alanine substitutions in a cluster of 7 residues located just C-terminal to the kinase domain (Fig. S2). This 7Ala substitution abolished binding of GST-BUR1(351-602) to Ser5P peptides (Fig. 2B-II), implicating these conserved residues in CTD binding. We found no obvious sequence similarity between the BUR1 CID and other known CIDs (Meinhart et al., 2005), suggesting that BUR1 contains a distinct CTD binding motif.

We asked next whether native BUR1-BUR2 complex can bind to Ser5P. TAP-tagged BUR1 proteins were purified on IgG resin, removing the protein A moiety with TEV protease. The resulting proteins, retaining the calmodulin binding domain (CBD), were tested for binding to CTD peptides. Full-length BUR1-CBD bound to untreated, but not to CIP-treated, Ser5P peptides (Fig. 2E), nor to Ser2P peptides (Fig. 2F). C-terminally truncated protein BUR1 (1-502)-CBD did not bind Ser5P nor unphosphorylated peptides (Fig. 2E). Equal amounts of BUR2-myc co-purified with full-length BUR1-CBD and a C-terminally truncated protein lacking residues 393-657 (previously dubbed *bur1-CΔ*) (Fig. 2G), confirming that the C-terminal half of BUR1 is dispensable for interaction with BUR2 (Keogh et al., 2003). These results indicate that native BUR1/BUR2 interacts with the Ser5-phosphorylated CTD dependent on the BUR1 CID.

The *bur1-CΔ* mutation confers cold-sensitive growth, the Bur$^-$ phenotype, and is synthetically lethal with *ctk1Δ*, and it reduces BUR1 recruitment to *ADH1* and *PMA1* by a factor of ≈2 (Keogh et al., 2003). Consistently, *bur1-CΔ* decreased GCN4-dependent occupancy of BUR1 at *ARG1*. This was clearly evident from reduced precipitation of *ARG1* sequences relative to their input levels in chromatin (Fig. 3A, cf. lanes 5-6 vs. 2-3), and after normalizing for BUR1 occupancies in *gcn4Δ* cells (Fig. 3B). ChIP signals for *ChrV* also were reduced in the *bur1-CΔ* strain (Fig. 3A), however, which might reflect a general reduction in chromatin association by bur1-CΔ protein. (The decrease in chromatin fragments specifically precipitated with bur1-CΔ would reduce the amount of ChrV fragments nonspecifically trapped in immune complexes.) Although normalization of the ChIP signals for binding at *ARG1* versus *ChrV* dampens the calculated reduction in BUR1 occupancy produced by the *-CΔ* mutation, it is still significantly reduced across *ARG1* (Fig. 3C). Because bur1-CΔ occupancy is more substantially reduced at the 5′ end of *ARG1*, it now peaks at the 3′ rather than 5′ end of the gene (Fig. 3C). Similar results were obtained for BUR2 in the *bur1-CΔ* background (Fig. 3D). These changes in BUR1 and BUR2 distribution across *ARG1* conferred by *bur1-CΔ* establish the

significance of the 5′ end enrichment of wild-type BUR1/BUR2 noted above. The *-CΔ* mutation does not reduce the steady-state level of BUR1 in cell extracts (data not shown). We conclude that *bur1-CΔ* impairs association of BUR1/BUR2 with elongating Pol II molecules, particularly those near the promoter. This fits with our finding that BUR1/BUR2 recruitment is enhanced by Ser5P, which also peaks at the 5′ end of *ARG1* (Fig. 1D). Hence, interaction of the BUR1 CID with Ser5 phosphorylated Pol II stimulates recruitment of the BUR1/BUR2 complex at promoter-proximal locations.

Recruitment of BUR1/BUR2 by Ser5P elevates Ser2 phosphorylation in vivo

Having shown previously that KIN28 stimulates recruitment of Paf1C to *ARG1*, we asked whether BUR1/BUR2 also promotes Paf1C recruitment as a CTD kinase by enhancing Ser5P formation. Instead, ChIP analysis with (H14) antibodies specific for Ser5P revealed that *bur2Δ* cells exhibit higher levels of Ser5P at *ARG1* (Fig. S3A). The *bur2Δ* mutant also displays slightly increased Pol II (RPB3) occupancy in the ORF (Fig, S3B), and after normalizing for this effect, the Ser5P:RPB3 ratio is found to increase, particularly near the 5′ end of *ARG1*, in *bur2Δ* cells (Fig. S3A-C). Interestingly, deletion of *CTK1*, the major Ser2P-CTD kinase, had a similar effect of increasing the Ser5P occupancy and Ser5P:RPB3 ratio at *ARG1* (Fig. S3D-F).

bur2Δ also resembles *ctk1Δ* in reducing SET2 recruitment and H3-K36Me3, except that in *bur2Δ* cells these defects are more pronounced at the 5′ ends of genes (Chu et al., 2007). Combining this fact with the sequence similarity between BUR1 and P-TEFb, and genetic similarities between CTK1 and BUR1, we reasoned that BUR1/BUR2 might promote Ser2 CTD phosphorylation, especially at the 5′ ends of genes. To test this, we conducted ChIP analysis of Ser2P at *ARG1* in WT, *gcn4Δ* and *bur2Δ* strains. Comparing WT and *gcn4Δ* cells revealed that GCN4-dependent Ser2P occupancy increases progressively from the TATA, through the ORF, to sequences downstream of *ARG1* in WT cells (Fig. 4A, lanes 6), as would be expected (Cho et al., 2001; Komarnitsky et al., 2000). Importantly, *bur2Δ* nearly eliminated Ser2P occupancy in the promoter and 5′ORF, and reduced Ser2P in the 3′ ORF (Fig. 4A, lanes 8), while having little effect on total Pol II (RPB3) occupancy (Fig. 4A, lanes 12 vs. 10). These results suggest that *bur2Δ* reduces the Ser2P content of elongating Pol II, particularly near the promoter, at *ARG1*. The ChIP signals for Ser2P at the *ChrV* sequences were also reduced in the *bur2Δ* strain (Fig. 4A, lanes 8 vs. 6), which could reflect a general reduction in chromatin association of Ser2-phosphorylated Pol II. Thus, normalization of the Ser2P ChIP signals at *ARG1* with those measured for *ChrV* dampens the reduction in GCN4-dependent Ser2P occupancy produced by *bur2Δ*. Nevertheless, the normalized GCN4-dependent occupancies of Ser2P and RPB3 still lead to significant decreases in Ser2P:RPB3 ratios at the 5′ and 3′ ends of *ARG1* in *bur2Δ* cells (Fig. 4B-D).

We found that eliminating CTK1, the major Ser2 CTD kinase, greatly reduces Ser2P levels across *ARG1* (Fig. 4A, lanes 7 vs. 6). Moreover, *ctk1Δ* has a much greater effect than *bur2Δ* on the Ser2P:RPB3 ratio at the 3′ end of *ARG1*, reducing it there by a factor of ~10 but only by a factor of ~3 at the 5′ end of the gene (Fig. 4D). These findings suggest that BUR1 and CTK1 make similar contributions to the Ser2P content of Pol II molecules located near the promoter, whereas CTK1 contributes the bulk of Ser2P at promoter-distal locations. The diminished Ser2P occupancy in *bur2Δ* cells does not result from decreased CTK1 occupancy at *ARG1* (Fig. 4F).

To provide evidence that BUR1/BUR2 is required for a proportion of Ser2P throughout the genome, we measured Ser2P levels in bulk RPB1 by Western analysis of WCEs. As expected, *ctk1Δ* strongly reduces total Ser2P (detected with antibody H5) while increasing hypophosphorylated RPB1 (detected with antibody 8WG16), while Ser5P is relatively unchanged (Fig. 4E). A darker exposure confirms that Ser2P is not abolished in *ctk1Δ* cells.

Importantly, *bur2Δ* also reduces Ser2P and increases hypophosphorylated RPB1, but the reduction in Ser2P was smaller than for *ctk1Δ* (Fig. 4E, *bur2Δ*). These findings support the idea that BUR1/BUR2 is required for Ser2 CTD phosphorylation of a subset of Pol II molecules or CTD repeats.

Having found that the *bur1-CΔ* mutation reduces recruitment of BUR1/BUR2 to the 5′ end of *ARG1* (Fig. 3A-D), we predicted that *-CΔ* should diminish the cellular Ser2P level. The Western analysis in Figs. 5A-B confirmed this expectation for *-CΔ* and also extended it to include the 7Ala substitution in the BUR1 CID that abolished binding to Ser5P peptides. These results imply that recruitment of BUR1/BUR2 to the 5′ ends of genes by association of the BUR1 CID with Ser5P enhances Ser2 CTD phosphorylation in bulk Pol II.

CTK1 and BUR1/BUR2 make additive contributions to Ser2P on elongating Pol II in vivo

From quantification of the Western data in Fig. 4E, we calculated that *ctk1Δ* reduces total Ser2P by ~90% (Fig. S4A). Thus, one might predict that BUR1/BUR2 would contribute only ~10% of the total Ser2P, whereas we calculated a 50% reduction in Ser2P for *bur2Δ* cells (Fig. S4A). A similar paradox was evident in the ChIP data above when considering the relative contributions of CTK1 and BUR1/BUR2 to Ser2P at the 3′ end of *ARG1* (Fig. 4A-B). It is possible that a proportion of Ser2P in the extract or chromatin is below the detection limit of Western and ChIP analysis, leading us to overestimate the contribution of CTK1 to Ser2P formation (see Fig. S4B for details). While this might explain some of the discrepancy, it appears that BUR1/BUR2 also contributes indirectly to Ser2P by stimulating CTK1 function. Assuming that BUR1/BUR2 is a Ser2 CTD kinase, its phosphorylation of a limited number of CTD repeats might enhance the ability of CTK1 to phosphorylate Ser2 throughout the CTD, so that *bur2Δ* would reduce the functions of both Ser2 CTD kinases. A third possibility, suggested previously (Keogh et al., 2003), is that *bur2Δ* reduces Ser2P only indirectly by reducing the amount of elongating Pol II available for Ser2 phosphorylation by CTK1. According to this last explanation, inactivating BUR1/BUR2 should have no effect on Ser2P levels in a *ctk1Δ* background. This prediction has been difficult to test because *bur1* Ts^- and *bur2Δ* mutations are synthetically lethal with *ctk1Δ* (Keogh et al., 2003;Murray et al., 2001;Xiao et al., 2007). Hence, we constructed an analog-sensitive (*as*) allele, harboring a Gly substitution of residue Leu-149 in the kinase domain (Bishop et al., 2001), to permit chemical inhibition of BUR1 kinase activity in a *ctk1Δ* background.

The *bur1-as* mutation renders the autokinase activity of immunopurified BUR1/BUR2 highly sensitive to the ATP analog NM-PP1, with essentially complete inhibition at 0.5 μM NM-PP1, while the activities of WT BUR1/BUR2 and CTK1 were unaffected by much higher analog concentrations (Fig. S5). Whereas growth of *bur1-as CTK1* cells is insensitive to high concentrations of inhibitor, NM-PP1 strongly inhibits growth of a *bur1-as ctk1Δ* double mutant on solid medium (Fig. 5C) and doubled the cell division time in liquid medium when added at 20 μM (data not shown). This fits with the idea that CTK1 and BUR1/BUR2 kinase functions are partially redundant in vivo. The fact that NM-PP1 does not inhibit growth of the *bur1-as* single mutant, even though *BUR1* is essential, suggests that NM-PP1 does not fully inactivate BUR1 kinase activity in vivo.

Similar to our findings on *bur2Δ*, treatment of *bur1-as* cells with NM-PP1 reduced Ser2P in bulk RPB1 and increased the amount of hypophosphorylated RPB1 (Fig. 5D, lanes 11-12 vs. 9-10 for Ser2P and RPB1 panels; quantification in Fig. 5E). Nevertheless, *ctk1Δ* still elicits a greater decrease in Ser2P levels compared to chemical inhibition of the *bur1-as* single mutant (Fig. 5D, lanes 11-12 vs. 7-8). Importantly, NM-PP1 treatment of the *bur1-as ctk1Δ* double mutant led to a further decline in Ser2P compared to the *ctk1Δ* single mutant, which became evident after increasing the amount of extract loaded per lane to facilitate detection of low-level Ser2P in *ctk1Δ* cells (Fig. 5F). These results provide direct evidence that BUR1 promotes

Ser2P formation, at least partly, by a mechanism independent of CTK1. ChIP analysis of the *bur1-as* mutant produced results consistent with those presented above for *bur2Δ* cells. Treating *bur1-as*, but not WT, cells with NMPP1 reduced Ser2P occupancy at *ARG1* (Fig. S6A-B) to an extent that cannot be explained by decreased Pol II occupancy (Fig. S6C-E). Thus, chemical inhibition of BUR1 lowers the Ser2P content of elongating Pol II molecules associated with *ARG1* coding sequences.

We sought to eliminate by an independent approach the possibility that inhibition of BUR1 reduces Ser2P levels only by decreasing the pool of elongating Pol II. We first used H14 antibodies to immunoprecipitate Ser5-phosphorylated RPB1, which should represent Pol II molecules engaged in promoter clearance or elongation, and probed them with H5 antibodies for Ser2P. The *ctk1Δ* mutation lowers the yield of Ser2P in H14-immunoprecipitated RPB1, in the presence or absence of NM-PP1 (Fig. 6A, Ser2P panel, cf. lanes 11-12 vs. 7-8 and 23-24 vs 19-20). This fits with the prediction that many Pol II molecules phosphorylated on Ser5 also contain Ser2P, and the expectation that CTK1 contributes to this Ser2 phosphorylation (Phatnani and Greenleaf, 2006;Wood and Shilatifard, 2006). Importantly, the analog-treated *bur1-as* single mutant also shows reduced Ser2P in H14-immunoprecipitated RPB1, but only when treated with NM-PP1 (Fig. 6A, Ser2P, lanes 21-22 vs. 19-20 and 9-10 vs. 7-8). As expected, Ser2P is essentially undetectable in RPB1 immunoprecipitated with 8WG16 antibodies specific for hypophosphorylated CTD (Fig. 6B). By the same approach, we found that *bur2Δ* also reduces the Ser2P content of immunoprecipitated RPB1 phosphorylated on Ser5 (Fig. S7). It could be argued that inactivation of BUR1/BUR2 reduces Ser2P in Pol II phosphorylated on Ser5 indirectly by preventing promoter escape by Pol II. This seems unlikely, however, considering that *bur2Δ* does not reduce the occupancy of Ser5P in the *ARG1* coding sequences (Fig. S3A). Hence, CTK1 and BUR1 both contribute to Ser2P in elongating Pol II molecules hyperphosphorylated on Ser5.

The reduction of Ser2P in immunoprecipitated Ser5-phosphorylated RPB1 produced by *ctk1Δ* is only slightly greater than that conferred by *bur1-as* in the presence of NM-PP1, whereas *ctk1Δ* has a much greater effect than *bur1-as* on Ser2P in bulk RPB1 (Fig. 6A; compare Ser2P signals in lanes 23-24 vs. 21-22 with lanes 17-18 vs. 15-16.) These comparisons suggest that BUR1 makes a larger contribution to Ser2 phosphorylation of Pol II molecules hyperphosphorylated on Ser5 compared to hypo- or unphosphorylated Pol II. By contrast, CTK1 makes a proportionately larger contribution to Ser2 phosphorylation of Pol II that is hypophosphorylated on Ser5.

Discussion

We have provided strong evidence that BUR1/BUR2 is required for high-level Ser2 phosphorylation in vivo, and that it contributes a proportion of Ser2P independently of the major Ser2 CTD kinase CTK1, particularly near promoters. We found that impairing BUR1 activity by eliminating BUR2 or chemically inhibiting the *bur1-as* product reduces Ser2P with an attendant increase in hypophosphorylated RPB1 in bulk Pol II. The *bur2Δ* and *bur1-as* mutations also decrease Ser2P occupancy in the *ARG1* coding sequences without a commensurate reduction in occupancy of elongating Pol II itself. BUR1/BUR2 makes a smaller contribution than CTK1 to the overall level of Ser2P in bulk Pol II. However, *bur2Δ*, *bur1-as* and *ctk1Δ* have comparable effects in reducing Ser2P at the 5′ end of *ARG1*, whereas *ctk1Δ* evokes a stronger decrease in Ser2P at the 3′ end of the gene. Moreover, *bur2Δ*, *bur1-as* and *ctk1Δ* all conferred strong reductions in Ser2P in the pool of bulk Pol II hyperphosphorylated on Ser5 (immunoprecipitated with H14 antibodies). These results suggest that BUR1/BUR2 makes a substantial contribution to Ser2P in Pol II molecules already phosphorylated on Ser5 and located near the promoter, whereas CTK1 is responsible for the majority of Ser2P and is the predominant Ser2 CTD kinase distal to the promoter.

Although a decrease in Ser2P at *PMA1* was noted previously in *bur1-23* cells, it was attributed to decreased occupancy of Pol II itself at this gene. In addition, while Ser2P in bulk Pol II was reduced in a *bur2Δ* strain, this was judged to be an indirect consequence of lower levels of elongating Pol II (Keogh et al., 2003). Our findings suggest that BUR1/BUR2's contribution to Ser2P is not limited to its role in producing elongating Pol II molecules as substrates for CTK1. First, Pol II occupancy in the *ARG1* ORF is not reduced in our *bur2Δ* strain, such that the Ser2P:RPB3 ratio at this gene is diminished in this mutant. Second, the Ser2P content of Pol II molecules phosphoryated on Ser5P, which should represent Pol II molecules engaged in elongation, is reduced in both *bur1-as* and *bur2Δ* mutants. Most importantly, inactivation of the *bur1-as* mutant in *ctk1Δ* cells eliminated the residual Ser2P in bulk Pol II that remains after eliminating CTK1 alone, proving that BUR1 can promote Ser2P formation independently of CTK1.

Combining these findings with the sequence similarity of BUR1 to P-TEFb, and genetic similarities between *BUR1* and *CTK1*, leads us to propose that BUR1/BUR2 functions as a Ser2 CTD kinase in vivo. Nevertheless, a proportion of the decline in Ser2P evoked by *bur2Δ* and *bur1-as* mutations, at least at promoter-distal locations, probably results indirectly from reduced phosphorylation by CTK1. This follows from our finding that *ctk1Δ* eliminates ≈90% of the detectable Ser2P in bulk Pol II, yet inactivating BUR1/BUR2 eliminates much more than 10% of the total Ser2P. One possibility is that Ser2 phosphorylation of a limited number of CTD repeats by BUR1/BUR2 near the promoter can enhance the ability of CTK1 to phosphorylate Ser2 throughout the CTD, and counteract the Ser2P phosphatase, as elongation proceeds downstream. This hypothesis is difficult to test in vitro because steady-state kinetic analysis of CTK1 phosphorylation of the full-length CTD is problematic (Jones et al., 2004), and it would impossible at present to reconstitute the extent or pattern of partial CTD phosphorylation by BUR1/BUR2 that prevails in vivo. It is also possible that phosphorylation of other, unknown substrates of BUR1/BUR2 indirectly stimulates Ser2P phosphorylation by CTK1 in vivo.

Our findings that *bur2Δ* and *bur1-as* produce a substantial decrease in Ser2P at the 5′ end of *ARG1*, and in Pol II hyperphosphorylated on Ser5, fit with our observations that BUR1/BUR2 occupancy peaks near the *ARG1* and *GAL1* promoters, and our discovery that recruitment of BUR1/BUR2 is stimulated by the Ser5 CTD kinase KIN28. We discovered that the C-terminal half of BUR1 contains a CID capable of binding Ser5P peptides in vitro. Removing this CID from BUR1 by the *−CΔ* mutation preferentially reduces BUR1/BUR2 occupancies at the 5′ end of the *ARG1* ORF, so that they peak in the 3′ end of the ORF. The *−CΔ* and *-7Ala* substitutions in the BUR1 CID also reduce the level of Ser2P in bulk Pol II. Hence, we propose that binding of the BUR1 CID to Ser5P, generated by KIN28, stimulates BUR1/BUR2 recruitment to the 5′ end of the gene (Fig. 7A), enhancing its ability to phosphorylate Ser2 early in the elongation cycle (Fig. 7B). We envision a cascade of CTD phosphorylation, wherein Ser5 phosphorylation by KIN28 enhances Ser2 phosphorylation by BUR1/BUR2 in the same or adjoining CTD repeats of promoter-proximal Pol II, which stimulates or gives way to Ser2 phosphorylation by CTK1 further downstream in the coding sequences (Fig. 7C).

In addition to stimulating BUR1/BUR2 recruitment, Ser5 phosphorylation by KIN28 might also enhance the ability of BUR1/BUR2 to phosphorylate Ser2, as shown previously for CTK1 (Jones et al., 2004). Our purified BUR1/BUR2 does not phosphorylate Ser5-phosphorylated or unphosphorylated CTD peptides of 4 heptad repeats under conditions where it phosphorylates a GST-CTD substrate with the native 26 repeats (data not shown). While this is an interesting observation for future study, it precluded our ability to determine if Ser5P enhances Ser2 phosphorylation by BUR1/BUR2 in vitro.

Most *bur1* mutations confer 6-AU sensitivity and are synthetically lethal with *spt5-194* and *ctk1Δ*, but *bur1-CΔ* is resistant to 6-AU and confers only a slight growth defect in the *spt5-194* background (Keogh et al., 2003). This suggests that decreasing BUR1/BUR2 recruitment to the Ser5-phosphorylated CTD by the *-CΔ* mutation has a modest effect on elongation, which agrees with our finding that Ser2P levels are only moderately reduced in the *bur1-CΔ* mutant. However, *bur1-CΔ* is synthetically lethal with *ctk1Δ*, which can now be explained by proposing that the moderate decrease in Ser2P levels conferred by *bur1-CΔ* is intolerable in the absence of CTK1. The same explanation can be extended to the *bur1-as* mutant, which produces a moderate decrease in Ser2P levels in *CTK1* cells and strongly impairs growth only in the *ctk1Δ* background.

Considering that *BUR1* is essential, it may seem surprising that the *bur1-as* mutant has almost no growth defect at high concentrations of NM-PP1, even though much lower NM-PP1 concentrations eliminate bur1-as/BUR2 kinase activity in vitro. We presume that NM-PP1 does not completely inhibit the *bur1-as* product in vivo, and that low-level kinase activity is sufficient for viability. Supporting this idea, substitution of Thr-240 in the BUR1 activation loop nearly destroys BUR1 kinase activity in vitro (Keogh et al., 2003; Yao and Prelich, 2002), but has little effect on growth in otherwise WT cells (Yao and Prelich, 2002) and confers only slow growth in the *ctk1Δ* background (Keogh et al., 2003). On the other hand, point mutations in conserved residues of the *BUR1* kinase domain that likewise abolish kinase activity in vitro are lethal, presumably because they completely eliminate kinase function in vivo.

Our conclusion that BUR1/BUR2 plays an important role in Ser2 phosphorylation of promoter-proximal Pol II provides new insights into the observation that BUR2 promotes H3-K36Me3 formation, especially near the 5′ ends of constitutively expressed genes (Chu et al., 2007). We made a similar observation for induced *ARG1*, finding that H3-K36Me3 occupancy is reduced by inactivation of the *bur1-as* product near the 5′ end of the gene (Fig. S8). Ser2 phosphorylation by CTK1 stimulates H3-K36 trimethylation by SET2 in downstream coding sequences (Kizer et al., 2005; Krogan et al., 2003b; Li et al., 2003; Xiao et al., 2003). Hence, our proposal that BUR1/BUR2 phosphorylates Ser2 on promoter-proximal Pol II molecules provides a possible explanation for the role of BUR2 in stimulating H3-K36Me3 formation near promoters (Fig. 7B). Considering that SET2 contains a CID that interacts preferentially with CTD peptides doubly phosphorylated on Ser2 and Ser5 (Kizer et al., 2005), BUR1/BUR2-mediated Ser2 phosphorylation of CTD repeats already phosphorylated on Ser5 should enhance SET2 function near the promoter. The effect of *bur2Δ* in reducing H3-K36Me3 formation was not limited to the 5′ ends of the *PYK1* and *FLO8* genes (Chu et al., 2007), which fits with our finding that *bur2Δ* reduces Ser2P levels throughout the *ARG1* ORF, and only makes a proportionately greater contribution at the 5′end.

Our ChIP analysis suggests that the occupancies of myc-BUR1, myc-BUR2 and myc-CTK1 do not vary greatly across the *ARG1* gene, with BUR1/BUR2 moderately exceeding CTK1 at the 5′ end owing to the BUR1 CID (cf. results in Figs. 1, 3, and 4). Thus, the fact that BUR1/BUR2 and CTK1 make roughly equal contributions to Ser2P formation at the 5′ end of *ARG1* might be explained quite simply by proposing that BUR1/BUR2 and CTK1 have similar kinase activities, as well as similar occupancies, near the promoter. On the other hand, the much greater contribution of CTK1 to Ser2P at the 3′end of *ARG1* seems to imply a change in kinase activity between the 5′ and 3′ ends of *ARG1* for BUR1/BUR2, CTK1, or both. For example, CTK1 could become more active as elongation proceeds, perhaps owing to the stimulatory effect of BUR1/BUR2 on CTK1 function deduced from our experiments, or to some other modification of the CTD. There is evidence that transient accumulation of H2B-Ub impedes CTK1 recruitment during *GAL1* induction (Wyce et al., 2007), leading us to consider whether this mechanism could have a role in reducing CTK1 activity near the

ARG1 promoter. However, it was reported that H2B-Ub accumulates transiently across the *GAL1* ORF, not only at the 5′end. And as noted above, CTK1 occupancy is not substantially lower at the 5′ versus 3′ end of *ARG1* (Fig. 4F), at least after 30 min of induction when our ChIP measurements were made.

Alternatively, BUR1/BUR2 might become less functional as transcription proceeds downstream. It could be proposed that a reduction in Ser5P distal from the promoter would diminish BUR1/BUR2 activity towards the 3′ end. This may be unlikely, however, because Ser5P remains quite high at the 3′ end of *ARG1* (Fig. S3). In fact, it is uncertain whether Ser5P levels, or only the reactivity of RPB1 to Ser5P-specific (H14) antibodies, declines as elongation proceeds (Phatnani et al., 2004). In addition, Ser2P formation by CTK1 is highly stimulated by Ser5P (Jones et al., 2004), so that loss of Ser5P would likewise reduce CTK1 function towards the 3′ end of the gene. Uncovering the mechanisms responsible for the greatly different contributions of BUR1/BUR2 and CTK1 to Ser2P formation between the 5′ and 3′ ends of *ARG1* remains an important goal for future research.

Materials and Methods

Yeast strains and plasmids used are listed in Table S1 and Table S2, and their construction or sources are described in the Supplementary Information. ChIP experiments were conducted using primers and antibodies as described in the Supplementary Information. Western analysis of WCEs prepared by TCA precipitation, and coimmunoprecipitation assays on native WCEs were conducted as described in the Supplementary Information, as were CTD peptide binding assays using synthetic biotin-conjugated peptides purchased from AnaSpec and streptavidin-conjugated magnetic beads. Purification of GST fusions and BUR1-TAP proteins and yeast growth assays to test NMPP1 sensitivity also are described in the Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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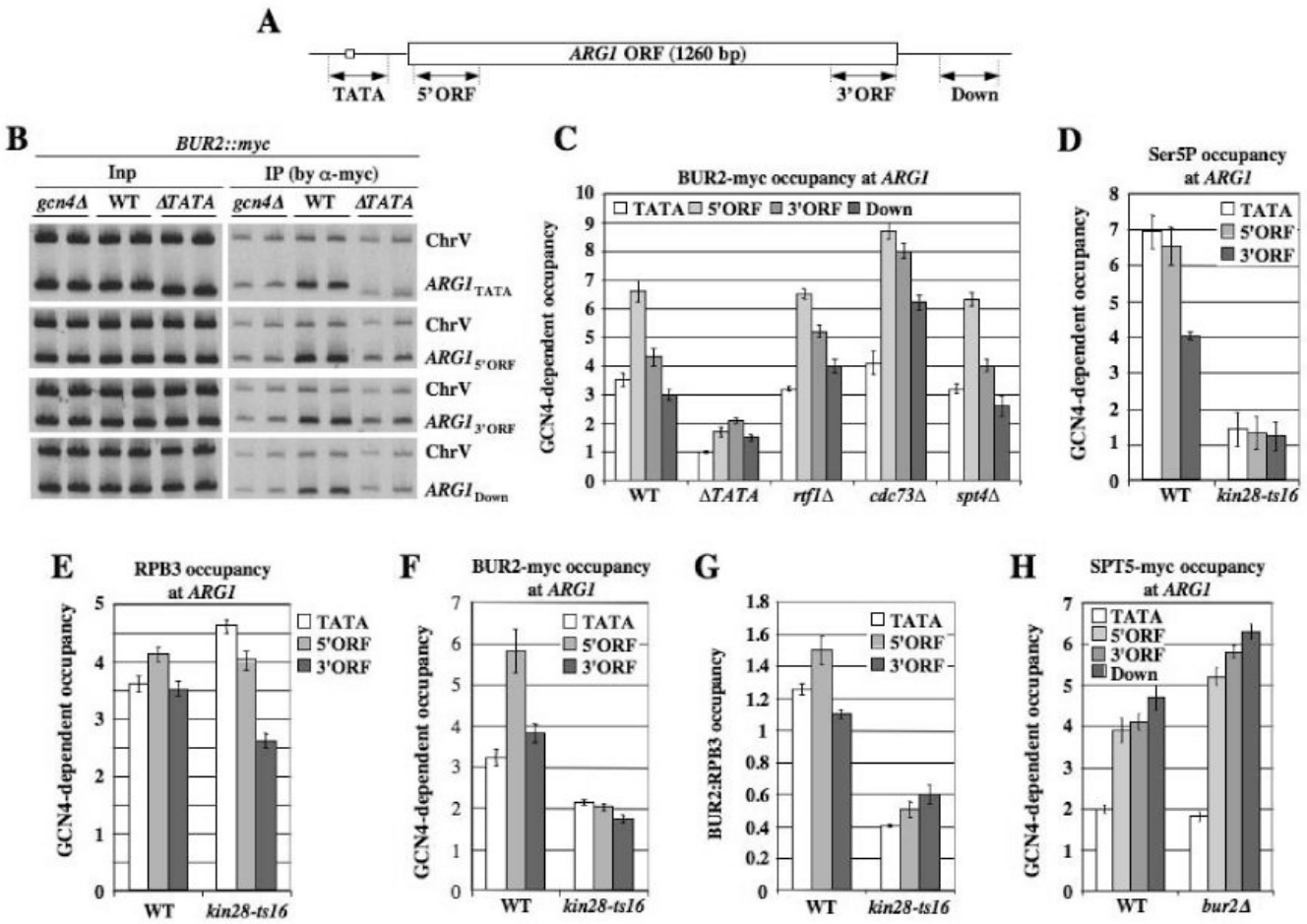

Fig. 1. Co-transcriptional recruitment of BUR2 to *ARG1* is enhanced by KIN28

(A) *ARG1* locus showing regions subjected to ChIP analysis. **(B, C)** ChIP analysis of BUR2-myc binding at *ARG1*. *BUR2::myc* strains with the indicated mutations (HQY1002, HQY1003, HQY1051, HQY1004, HQY1007, HQY1008) were cultured in SC medium lacking Ile and Val and treated with sulfometuron (SM) for 30 min to induce GCN4, then subjected to ChIP analysis with myc antibodies. DNA extracted from immunoprecipitates (IP) and input chromatin (Inp) samples was subjected to PCR in the presence of [^{33}P]-dATP with the appropriate primers to amplify radiolabeled fragments of *ARG1* shown in (A) or a control fragment (ChrV). PCR products were resolved by PAGE and visualized by autoradiography, with representative results shown in (B), and quantified with a phosphorimager. The ratios of *ARG1* to ChrV signals in IP samples were normalized for the corresponding ratios for Inp samples. The resulting values for the *GCN4* strains were normalized to the corresponding values for the *gcn4*Δ strain to yield GCN4-dependent occupancies plotted in (C). **(D-G)** *BUR2::myc* strains (HQY1052, HQY1055, HQY1053) were grown at 25°C to OD_{600} of ~0.6, transferred to 37°C for 30 min and treated with SM for another 30 min at 37°C. ChIP analysis was conducted as described above using H14 antibody (D), RPB3 antibody (E), or myc antibody (F). Values for BUR2-myc in (F) were normalized to those for RPB3 in (E) to calculate the ratios in (G). (**H**) *SPT5::myc* strains (HQY971, HQY973, HQY1040) were subjected to ChIP analysis as above. The error bars in this and all subsequent figures correspond to standard errors of the mean.

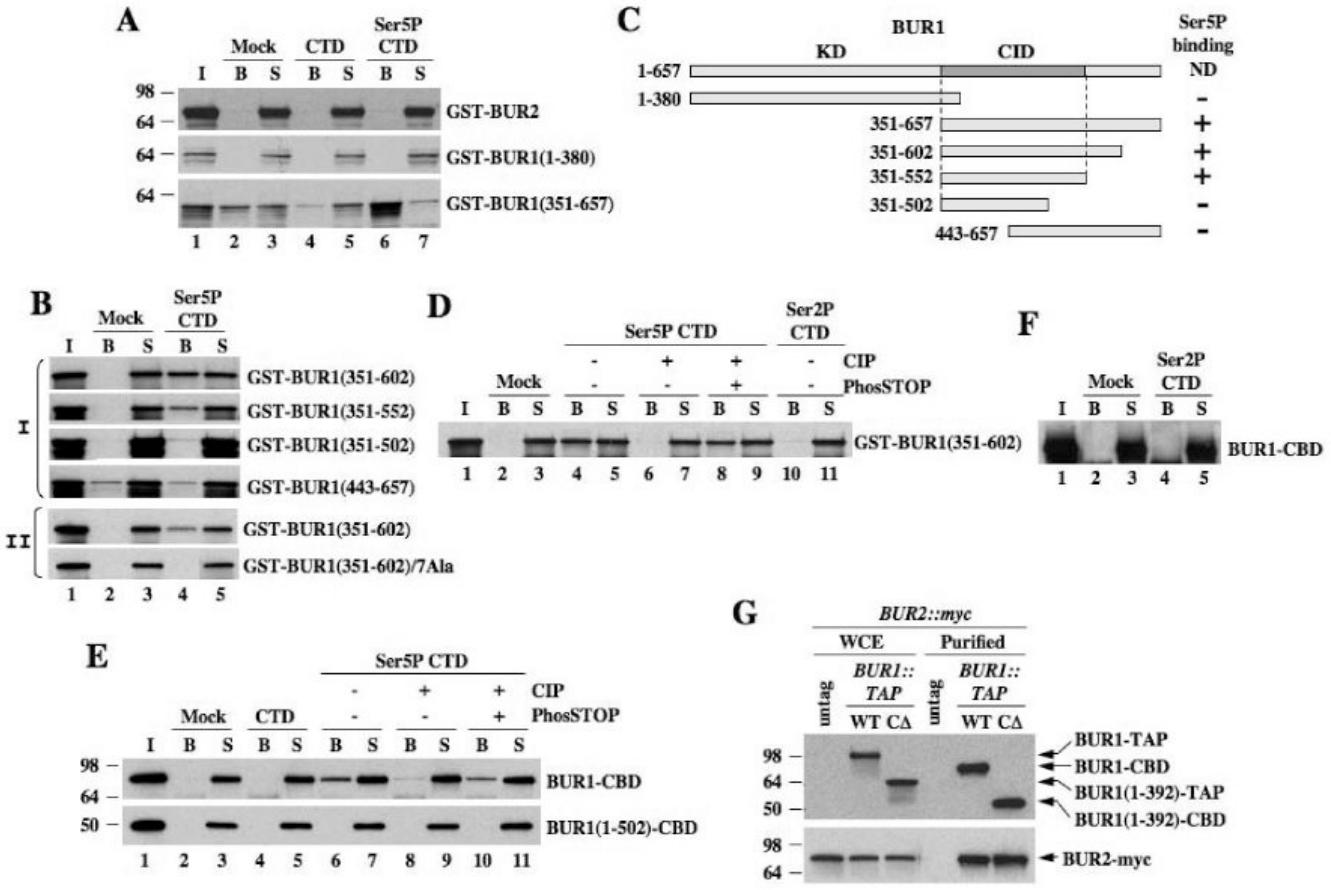

Fig. 2. BUR1 binds to Ser5P-CTD peptides

(A-D) Biotinylated CTD peptides (1.5μg) phosphorylated on Ser5 (Ser5P-CTD) or unphosphorylated (CTD) were adsorbed to streptavidin-coated magnetic beads. Recombinant GST-BUR2 or GST-BUR1 fragments were incubated with beads alone (Mock) or beads bearing peptides at 4°C. Bound (B) and unbound proteins in supernatant (S) were subjected to SDS-PAGE and Western analysis with myc antibody. (C) Schematic summarizing results in (A-B). (**E-F**) BUR1-CBD and BUR1(1-502)-CBD purified from yeast were used for peptide binding assays as above and detected using anti-TAP antibodies. In (D-E), immobilized peptides were treated with CIP in the presence or absence of PhosSTOP prior to incubation with proteins. (**G**) BUR1-TAP and BUR1-CΔ-TAP were purified from *BUR2::myc* strains and subjected to SDS-PAGE and Western analysis, along with the starting extracts (WCE), using antibodies against TAP (upper panel) and myc (lower panel).

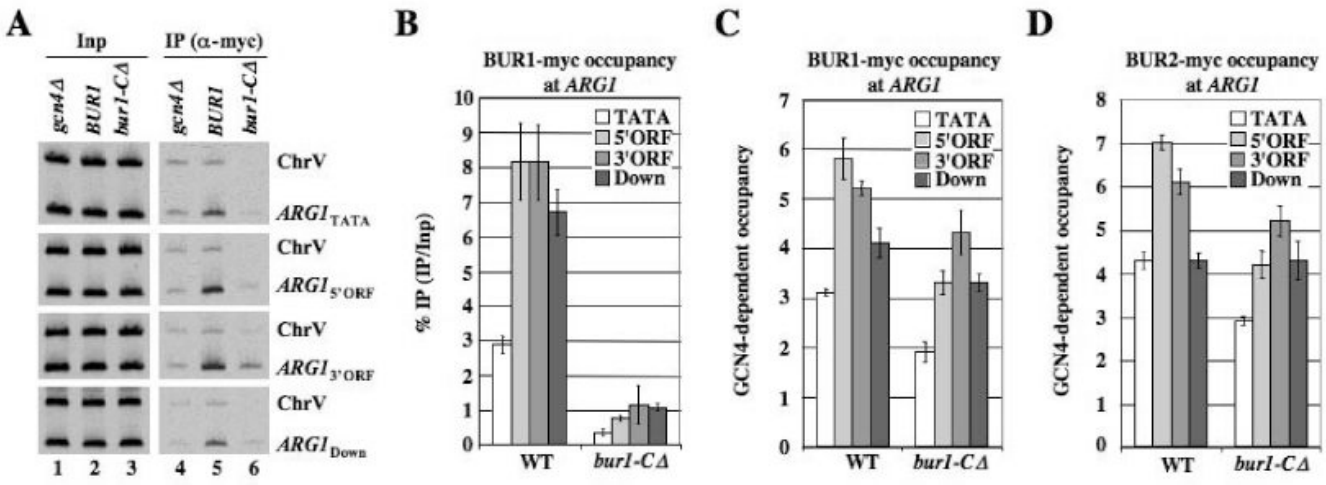

Fig. 3. The BUR1 CID enhances BUR1/BUR2 recruitment to the *ARG1* 5′ ORF in vivo

(**A-C**) *BUR1::myc* strains (HQY1188, HQY1223, HQY1280) and (D) *BUR2::myc* strains (HQY1002, HQY1003, HQY1268) were subjected to ChIP analysis as described in Fig.1, with representative results shown in (A), and GCN4-dependent occupancies plotted in (C-D). (B) gives the ratio of IP to Inp signals for *ARG1*, normalized to the corresponding values for the *gcn4Δ* strain, without normalizing for the ChrV signals.

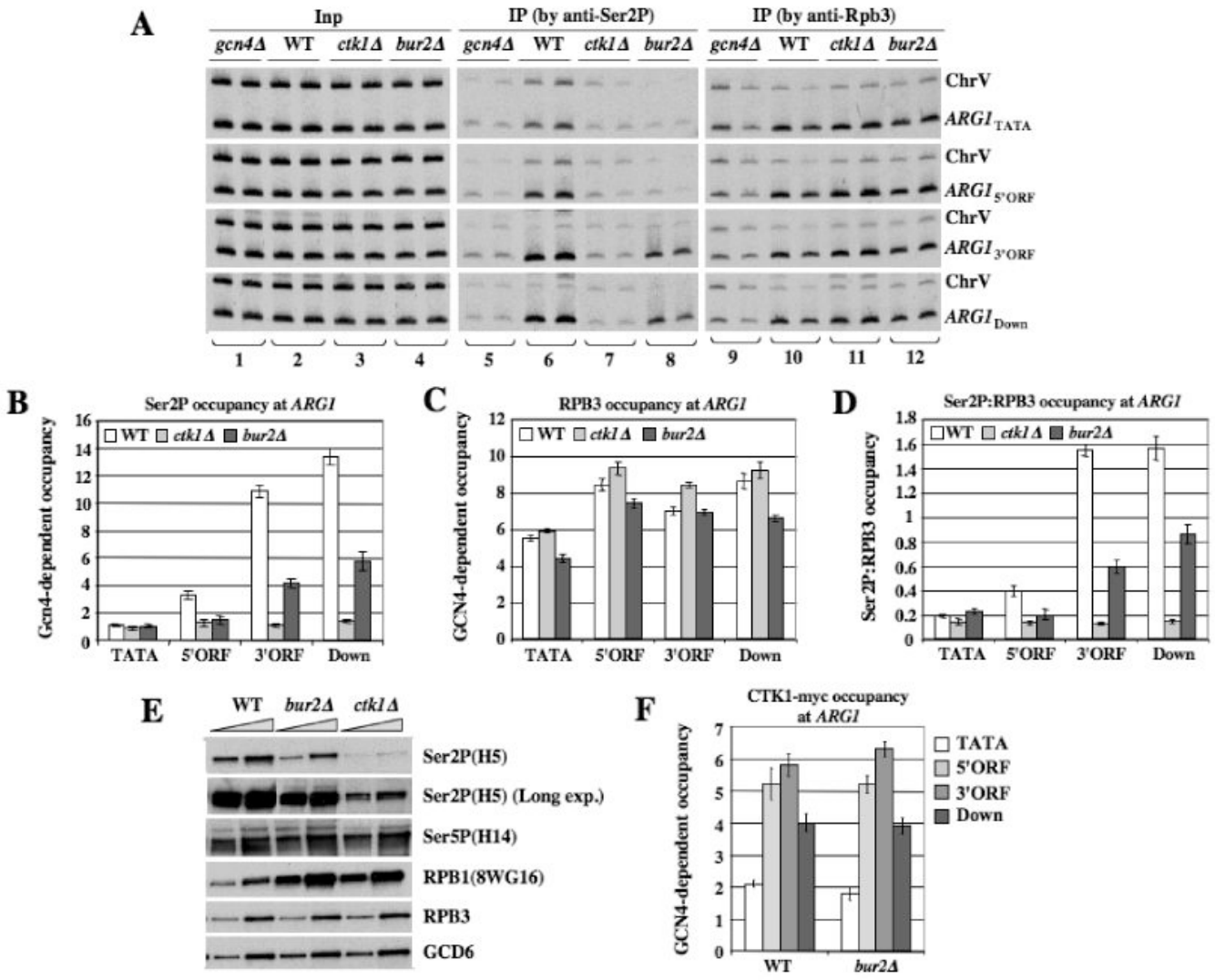

Figure 4. *bur2*Δ reduces Ser2P occupancy at the 5′ end of *ARG1*

(**A-D**) Strains with the indicated mutations (249, BY4741, 7028, HQY1038) were subjected to ChIP analysis as described in Fig.1 using antibodies for Ser2P or RPB3, with representative results shown in (A). (B-C) GCN4-dependent Ser2P or RPB3 occupancies were calculated as described in Fig. 1 (ie. normalizing for ChrV signals) and ratios of these occupancies are plotted in (D). (**E**) WCEs of strains with the indicated mutations prepared under denaturing conditions (TCA extraction) were subjected to Western analysis with the indicated antibodies against Ser2P, Ser5P, or hypophosphorylated RPB1 (8WG16), or against RPB3 or GCD6. (**F**) *CTK1::myc* strains (HQY1010, HQY1012, HQY1108) were subjected to ChIP analysis as in Fig.1.

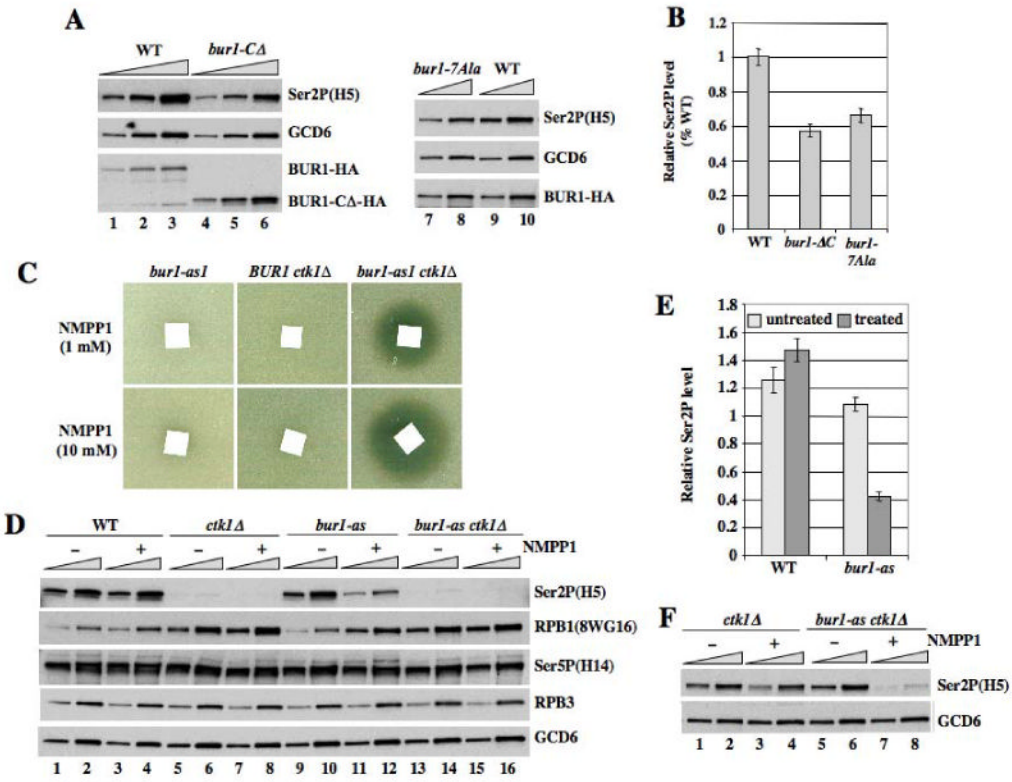

Fig. 5. Chemical inactivation of the *bur1-as* product reduces Ser2P in bulk Pol II

(**A-B**) Western analysis of WCEs prepared under denaturing conditions from WT, *bur1-ΔC*, and *bur1-7Ala* strains (HQY1269, HQY1270, H1299), using antibodies against the indicated proteins. Quantification of results for Ser2P normalized for GCD6 is shown in (B). **(C)** Strains HQY1190, HQY1221 and HQY1220 with the indicated mutations were plated in soft agar containing 3 μl of 1 mM or 10 mM NM-PP1 applied to a filter paper square. **(D-F)** Strains from (C) and HQY1223, with the indicated mutations, untreated (-) or treated (+) with NMPP1, were subjected to Western analysis as in (A-B). 12.5-25-fold amounts of WCEs for the *ctk1*Δ and *bur1-as ctk1*Δ strains in (D) were examined in (F).

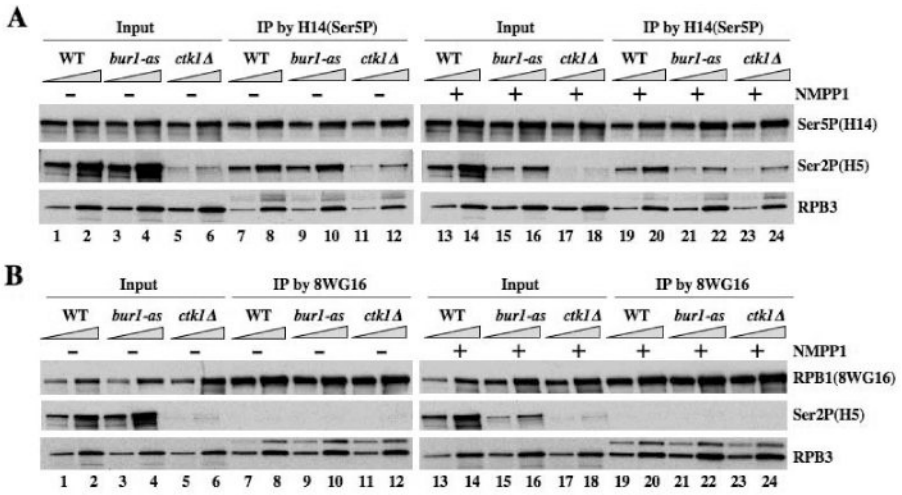

Fig. 6. Inactivation of BUR1 reduces Ser2P in elongating Pol II

(**A-B**) WCEs from strains with the indicated mutations (HQY1223, HQY1221, HQY1190), untreated (-) or treated (+) with NMPP1, were prepared under non-denaturing conditions and immunoprecipitated with H14 (A) or 8WG16 antibodies (B) and immune complexes were probed with antibodies against the indicated proteins.

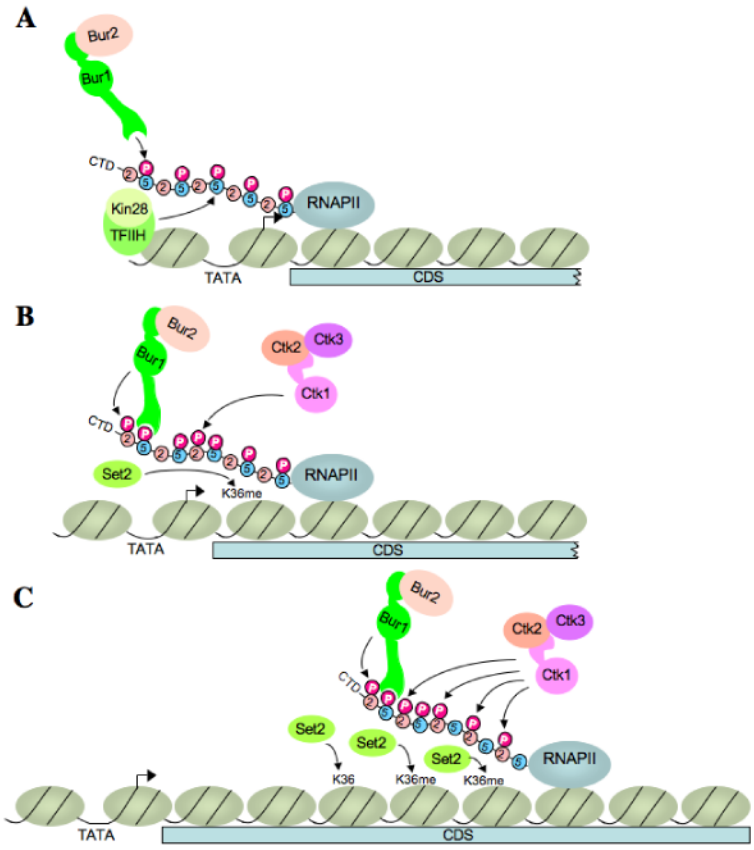

Fig. 7. Schematic model of Ser5P-stimulated BUR1/BUR2 recruitment and differential contributions of BUR1/BUR2 and CTK1 to Ser2P and H3-K36Me3 formation at 5′ and 3′ ends of a coding sequence

(**A**) BUR1/BUR2 is recruited to the CTD phosphorylated on Ser5 by KIN28 at or near the promoter. (**B**) Recruited BUR1/BUR2 phosphorylates Ser2 near the promoter, enhancing H3-K36Me3 formation by SET2. It is unknown whether Pol II pausing early in the coding sequence (CDS) is required to facilitate BUR1/BUR2 function. BUR1/BUR2 and CTK1 make roughly equivalent contributions to Ser2P formation at this location. (**C**) As elongation proceeds downstream, CTK1 makes an increasingly larger contribution to Ser2P formation and attendant H3-K36Me3 formation by SET2. Because the occupancies of both kinases remain high at the 3′ end, either CTK1 becomes more active, or BUR1/BUR2 activity declines, as elongation proceeds. See text for more details.