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Bur1 Kinase Is Required for Efficient Transcription Elongation by RNA Polymerase II

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The *Saccharomyces cerevisiae* cyclin-dependent kinase (CDK) Bur1 (Sgv1) may be homologous to mammalian Cdk9, which functions in transcriptional elongation. Although Bur1 can phosphorylate the Rpb1 carboxy-terminal domain (CTD) kinase in vitro, it has no strong specificity within the consensus heptapeptide YSPTSPS for Ser2 or Ser5. *BUR1* mutants are sensitive to the drugs 6-azauracil and mycophenolic acid and interact genetically with the elongation factors Ctk1 and Spt5. Chromatin immunoprecipitation experiments show that Bur1 and its cyclin partner Bur2 are recruited to transcription elongation complexes, cross-linking to actively transcribing genes. Interestingly, Bur1 shows reduced cross-linking to transcribed regions downstream of polyadenylation sites. In addition, *bur1* mutant strains have a reduced cross-linking ratio of RNA polymerase II at the 3′ end of genes relative to promoter regions. Phosphorylation of CTD serines 2 and 5 appears normal in mutant cells, suggesting that Bur1 is not a significant source of cotranscriptional Rpb1 phosphorylation. These results show that Bur1 functions in transcription elongation but may phosphorylate a substrate other than the CTD.

Synthesis of mRNA by RNA polymerase II (RNApII) requires transcription complex assembly, initiation, promoter clearance, elongation, and termination (22, 23). Each of these steps is a potential regulation point. Rpb1, the largest subunit of RNApII, plays a key role in gene expression (23, 27). In addition to its enzymatic activity, Rpb1 contains a conserved carboxy-terminal domain (CTD) that plays a central role in transcription (15). The *Saccharomyces cerevisiae* CTD contains 27 repeats of the consensus heptapeptide YSPTSPS (2, 13). During transcription, the CTD is subject to multiple phosphorylations by a series of cyclin-dependent kinases (CDKs). Phosphorylation occurs primarily on serine 2 (S2) and serine 5 (S5) of the heptapeptide (13).

Unphosphorylated Rpb1 is preferentially recruited to preinitiation complexes and is phosphorylated during the transition from initiation to elongation. An S5-phosphorylated form of CTD predominates at the promoter, with S2-phosphorylated CTD more prevalent in distal regions (11, 36). Hyperphosphorylated CTD serves as a docking site for complexes involved in mRNA maturation, including those involved in capping, splicing, and polyadenylation (12, 27, 46, 47). The CTD tail is near the mRNA exit channel of RNApII, providing the nascent transcript proximity to the processing machinery (14).

Four *S. cerevisiae* CDKs are reported to phosphorylate the CTD. An attractive theory to account for functional differences between these kinases invokes both different site preferences within the CTD and different temporal windows of activity (26, 36). Srb10 (the mammalian homologue is Cdk8) is a component of Mediator but may phosphorylate the CTD prior to preinitiation complex formation, thereby acting as a transcription inhibitor (20, 26). Srb10 also phosphorylates substrates other than the CTD (1, 3, 28, 50). Kin28 and its cyclin partner Ccl1 (homologous to mammalian cdk7/cyclin H) are subunits of the basal transcription factor TFIIH. Kin28 phosphorylates the CTD on S5 in the preinitiation complex. This phosphorylation event is important for recruitment of the capping machinery (6, 62). Ctk1 and Ctk2 are the catalytic and cyclin subunits of the CTDK1 complex (38, 41, 44, 70). The exact role of Ctk1 is unclear, but it associates with the transcription elongation complex and is necessary for phosphorylation of S2 during this stage of transcription (11). Deletion of the *CTK1* gene leads to a number of phenotypes and genetic interactions consistent with a role in elongation (38, 41, 44).

The fourth cyclin-dependent CTD kinase in yeast is Bur1 (also known as Sgv1). Ctk1 and Bur1 have equivalent levels of sequence similarity to mammalian Cdk9. Bur1 was identified in two independent selections: as a suppressor of the mating pheromone hyperadaptivity caused by the Gpa1(Val50) mutation (30), and as a mutation that increases transcription from a *SUC2* basal promoter lacking a upstream activation sequence (UAS) (bypass of UAS requirement) (57). Bur1 and Bur2 form a divergent CDK-cyclin pair (76). A number of recessive phenotypes have been ascribed to Bur1 mutants. The original *sgv1* mutant allele does not grow at high or low temperature and is sensitive to α-factor growth arrest (30). This mutant arrests as large unbudded cells at the nonpermissive temperature but can be rescued by overexpression of an activating *CLN3* mutant (*cln3-2*). Some Bur1 (*bur1-2*) and Bur2 mutant alleles (*bur2-1* and *bur2-2*) exhibit an Spt$^-$ phenotype, a sporulation defect, and sensitivity to 2% formamide or 15 mM caffeine (57, 76). There is one difference between the two genes: *bur1*Δ is reported to be lethal, while *bur2*Δ is viable (76).

Bur1 coimmunoprecipitates Rpb1 and phosphorylates the CTD on S5 in vitro (48). Genetic screens demonstrate that the *bur1-2* allele genetically interacts with Rpb1 CTD truncations, some *kin28* mutants, *ctk1*Δ, a catalytic mutant of the CTD

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source
YSB455	*MAT***a**/α *ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 lys2Δ202/lys2Δ202*	Lab strain
YSB725	*MAT*α *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202*	This study
YSB726	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202*	This study
YSB759	*MAT***a** *kin28Δ::LEU2 tfb1Δ::LEU2 ura3- leu2-3,112 trp1-1 his3- ade2-1(pRS314-haKIN28*; *pRS313-TFB1-6His*)	Keogh et al. (34)
YSB770	*MAT***a** *BUR1-(HA)$_3$::TRP1(KL) ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202*	This study
YSB772	*MAT***a** *CTK1-(HA)$_3$::TRP1(KL) ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202*	Cho et al. (11)
YSB776	*MAT***a** *SRB10-(HA)$_3$::TRP1(KL) ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202*	This study
YSB777	*MAT***a** *MED6-(HA)$_3$::TRP1(KL) ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202*	This study
YSB787	*MAT***a** *bur1Δ::HIS3 ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202(pRS316-BUR1)*	This study
YSB788	*MAT*α *bur1Δ::HIS3 ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202(pRS316-BUR1)*	This study
YSB789	*MAT***a** *bur1Δ::his3Δ::TRP1*/Kan^r *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202(pRS316-BUR1)*	This study
YSB797	*MAT***a** *bur1Δ::HIS3 ppr2Δ::hisG ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202(pRS316-BUR1)*	This study
YSB799	*MAT*α *bur1Δ::HIS3 suc2ΔUAS* (−1900/−390) *ura3-52 leu2Δ1 his3Δ200* or *HIS3 trp1Δ63 lys2-128δ(pRS316-BUR1)*	This study
YSB813	*MAT***a** *BUR2-(HA)$_3$::TRP1(KL) ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202*	This study
YSB855	*MAT***a** *bur1Δ::his3Δ::TRP1*/Kan^r *ctk1Δ::HIS3 ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202(pRS316-BUR1)*	This study
YSB856	*MAT***a** *bur1Δ::HIS3 spt5-194 ura3-52 leu2Δ1 his3Δ200 lys2-128δ(pRS316-BUR1)*	This study
FY121	*MAT***a** *suc2ΔUAS*(−1900/−390) *ura3-52 trp1Δ63 lys2-128δ his4-912δ*	Prelich and Winston (57)
GY100	*MAT***a** *suc2ΔUAS*(−1900/−390) *bur1-2 ura3-52 leu2Δ1 lys2-128δ his4-912δ*	Prelich and Winston (57)
GHY594	*MAT*α spt5-194 *ura3-52 his3Δ200 lys2-128δ*	Hartzog et al. (25)

phosphatase Fcp1 (*fcp1-110*), *spt4*Δ, *spt5-194*, and a deletion of the elongation factor TFIIS (*ppr2*Δ) (44, 48). However, no genetic interactions with *srb10*Δ were observed (44). Bur1 mutants also display sensitivity to the nucleotide analog 6-azauracil (6AU) (48). Although it affects transcription, Bur1 does not appear to be a component of preinitiation complexes (61). All of these results are consistent with, but do not prove, a role for Bur1 in transcription elongation.

To explore further the role of Bur1, a series of mutants were generated and analyzed. Like other CDKs, Bur1 is phosphorylated on the T-loop. In addition to the typical CDK kinase domain, Bur1 has an extended C-terminal region. Kinase activity is essential for Bur1 function. T-loop phosphorylation and the C-terminal region are not essential, but mutations of these features lead to distinct phenotypic patterns, suggesting separable roles for the two domains. In chromatin immunoprecipitation (ChIP) analysis, Bur1 and its cyclin partner Bur2 cross-link to coding regions of genes in a manner dependent upon transcription, but not on Bur1 kinase activity. Interestingly, cross-linking of Bur1 and Bur2 drops after RNApII transcribes through a polyadenylation site. Inactivation of Bur1 using temperature-sensitive mutants leads to a decrease in cross-linking of elongating RNApII, which is most pronounced towards the 3′ end of genes. This pattern is very similar to that seen in cells grown in the presence of 6AU. Significantly, cotranscriptional phosphorylation of CTD S2 and S5 are not strongly affected in the Bur1 mutants. Based on these results, we believe that the Bur1 kinase promotes elongation by phosphorylating a substrate other than the CTD.

MATERIALS AND METHODS

Yeast strains, genetic manipulations, and media. Yeast strains used in this study are listed in Table 1. All strains are in the S288C background. Parentheses indicate episomal plasmids. One copy of the *BUR1* gene was disrupted in the diploid strain YSB455 by one-step gene disruption with a *HIS3* marker. A derived haploid strain, YSB787, containing an episomal copy of the wild-type *BUR1* gene on a *URA3*-marked plasmid (pRS316-BUR1) was used for plasmid shuffling of *bur1* mutants. Yeast strains were transformed by the lithium acetate procedure (19). Standard methods for media preparation, mating, sporulation, and tetrad analysis were used (5, 21).

Plasmids. Yeast plasmids were based on the pRS series (67). For mutational analysis of Bur1, a 2.45-kb fragment containing the *BUR1* open reading frame (ORF) (no stop codon) and ≈400 bp of the 5′ promoter region was PCR amplified from *S. cerevisiae* genomic DNA using *Pfu* polymerase and oligos Bur1 5′*Sac*I(N) (GATCGAGCTCCCGAGAAATCAGCCGTTGG) and Bur1 3′FL Bam (CTAGGGATCCATATAGATCTGCAATATCACTATTTTGG). The resulting product was gel purified and cloned in frame with an hemagglutinin $(HA)_3$ epitope tag and SSN6 terminator at the carboxyl terminus. This product was transferred to pRS315 to create pRS315-BUR1-HA_3.

Point mutations in Bur1 (T70A, E107Q, D213A, T240A, and T240D) were generated using PCR-mediated site-directed mutagenesis (29, 34). Primer Bur1 5′*Sac*I(N) and the appropriate mutagenic primer (sequences available upon request) were used to amplify the 5′ end of the *BUR1* gene. The resulting PCR product was gel purified and utilized as a 5′ megaprimer in a second PCR with the 3′ primer Bur1 3′FL Bam. The resulting 2.45-kb amplified fragments were cloned into pRS315-$(HA)_3$-SSN6 such that the mutant protein was epitope tagged at the C terminus.

Epitope tagging at chromosomal loci. A PCR strategy was utilized to generate DNA fragments containing the $(HA)_3$ epitope and the *Kluyveromyces lactis TRP1* gene flanked by sequences from the 3′ end of the targeted gene. Inserts for homologous recombination were PCR amplified from pKL-TRP$(HA)_3$ as described previously (59) with the appropriate primers (sequences available upon request). Transformation and homologous recombination places the $(HA)_3$ epitope in frame at the carboxyl terminus of the targeted gene. Transformants were selected using the heterologous *TRP1* marker, and tagging was confirmed by immunoblotting. The Bur1, Bur2, Med6, Ctk1, and Srb10 proteins were tagged for this work.

Isolation of Bur1 temperature-sensitive alleles. *BUR1* was randomly mutagenized by PCR misincorporation using *Taq* polymerase and biased deoxynucleoside triphosphate concentrations (0.4 mM dGTP, dCTP, and dTTP and 0.1 mM dATP). The PCR fragment containing the *BUR1* gene was cotransformed into the Bur1 shuffling strain YSB787 together with the *Sac*II/*Xba*I-cut backbone of pRS315-Bur1-HA_3-SSN6. This gapped plasmid contains approximately 500 bp of 5′ and 3′ overlap with the PCR product, allowing recombination to occur in vivo. Colonies were selected on medium lacking leucine. After shuffling out the wild-type Bur1 plasmid with 5-fluoroorotic acid (5-FOA), cells were replica plated at 30 and 37°C to screen for temperature sensitivity. Plasmid DNA was rescued from temperature-sensitive (*ts*) candidates and retransformed to confirm plasmid linkage.

Seven alleles were chosen for further analysis: *bur1-23*, *-80* and *-85* (very strong *ts*), *bur1-35*, *-51* and *-65* (strong *ts*), and *bur1-78* (weak *ts*). Sequencing of the

ORFs showed each allele to contain multiple unique mutations as follows: *bur1-23* (Q258R, L326Q, L334Q, K366R, I375K, Y462C, A478V, and N509K); *bur1-80* (N5S, L155P, K374E, T379A, and N550Y); *bur1-85* (D31G, R83G, E95G, Q290L, S534P, R553G, N568D, K585Stop, and S616T); *bur1-35* (S45G, F70L, I147T, I200T, E316G, I387T, K470R, V482A, and K522R); *bur1-51* (R44G, V49A, A86V, I109T, G235S, and Y351H); *bur1-65* (Q177R, P301Q, H361R, K441 M, E454G, and N458D); *bur1-78* (M175V, L179S, L254F, K278E, and H361L).

Protein analysis and immunoprecipitations. Yeast whole-cell extracts were prepared by glass bead disruption of cells as described previously (34). Immunoblotting was performed using standard methods. $(HA)_3$-tagged proteins were immunoprecipitated from whole-cell extracts as indicated with 12CA5-protein A-complexed beads. Complexes were prepared by mixing 10 μl of protein A resin and 2 μl of 12CA5 ascitic fluid per sample in Tris-EDTA (TE; pH 8.0) and incubated with gentle rolling for 30 min at 4°C. Beads were pelleted by centrifugation, washed twice with 1 ml of TE (pH 8.0), and diluted 1:1 with TE (pH 8.0). A 10-μl aliquot of this mix was then added per sample. Protein input for each immunoprecipitation was as indicated, but in all cases samples were incubated with gentle rolling overnight at 4°C. Beads were then pelleted by centrifugation and washed three times in 1 ml of lysis buffer (20 mM HEPES [pH 7.6], 200 mM KOAc, 10% glycerol, 1 mM EDTA) prior to analysis.

In vitro CTD kinase assay. A recombinant glutathione *S*-transferase (GST)–HA–CTD fusion protein (see Fig. 4) or an HA-CTD protein cleaved by thrombin from the GST fusion (see Fig. 3) was phosphorylated with immunoprecipitated $(HA)_3$-tagged proteins and [γ^{32}P]ATP as described previously (34). Final concentration per 25-μl reaction volume was 20 mM HEPES-NaOH (pH 7.6), 7.5 mM magnesium acetate, 2 mM dithiothreitol, 100 mM potassium acetate, 2% glycerol, 25 μM ATP, 0.5 μl of [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear), 100 ng of GST-CTD. Reactions were incubated at room temperature for 30 min, resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and exposed to X-ray film.

Phenotypic analyses. For spotting analyses, cells were resuspended at 10^7/ml and subjected to 10-fold serial dilutions. A 20-μl aliquot of each dilution was spotted per plate. Strains were assayed for sensitivity to 6AU (10, 25, 50, or 75 μg/ml) or mycophenolic acid (MPA; 15 μg/ml). Growth was assayed at 48 or 72 h as indicated.

ChIPs. Chromatin immunoprecipitations (ChIPs) were performed essentially as described previously (36, 39) with minor modifications. A 250-ml volume of each strain was grown to an optical density at 600 nm (OD_{600}) of ≈0.6 in synthetic complete medium supplemented as necessary. Formaldehyde was added to a final concentration of 1% for 20 min, and the reaction was quenched by the addition of glycine to 240 mM. Cells were collected by centrifugation, washed twice with Tris-buffered saline, and lysed with glass beads in FA lysis buffer (50 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride). Chromatin was sheared by sonication such that the average fragment size was between 200 and 500 bp.

For immunoprecipitations, monoclonal antibodies were preincubated for 30 min at room temperature with protein A-agarose or protein G-Sepharose CL-4B beads as indicated and washed once with TE (pH 8.0). Chromatin solution was then added, and reactions were incubated overnight at 4°C. Immunoprecipitates were washed, protease treated, and de-cross-linked. Conditions for PCR were as described previously (36). PCR products were quantified by using a Fujix BAS 2040 PhosphorImager. The efficiency of amplification for each primer pair relative to a chromosome V no-ORF control pair was calculated from the input sample. This value was then used to normalize the specific signal obtained from each immunoprecipitation. Division of this normalized value by the background amplification of the no-ORF control gave a relative value (x-fold compared to the internal control) that allowed trend comparisons across samples to be performed. For IPs and PCRs from the same chromatin sample, variability was typically no more than 10 to 20% of the signal.

RESULTS

Analysis of Bur1 mutants. Bur1 has canonical motifs indicative of a CDK. Mutation of a number of conserved residues was performed to test the contribution of catalysis and regulatory phosphorylation to Bur1 activity. Analysis of the Bur1 protein sequence showed that the N-terminal region resembles CDKs. The C-terminal region (amino acids 392 to 657) has no significant homologues, and its function is unknown. Based on alignment with the human CDK2 structure (32, 63), Bur1 E107 is predicted to be part of the PSTAIRE loop within the catalytic site, and D213 is predicted to be involved in coordinating magnesium. There are two potential phosphorylation sites: T240 is predicted to be the site of an activating phosphorylation, while T70 corresponds to the site of an inactivating phosphorylation seen in some CDKs (reviewed in reference 68) (Fig. 1A).

Mutations in these residues were created in the context of an epitope-tagged Bur1. A truncation that removes amino acids 393 to 657 (CΔ) of Bur1 was also created. The mutants were transformed into a *bur1*Δ::HIS3 shuffling strain (Table 1), and expression of mutant proteins was confirmed by immunoblotting (Fig. 1B). Plasmid shuffling was then performed, and growth of the resulting strains was examined (Fig. 1C). The previously described *bur1-2* mutant (R311Δ) (57) was also tested. In contrast to previous reports describing *bur1*Δ as lethal, we found that the deletion strain was viable, albeit with extremely slow growth that rendered it essentially unusable (data not shown). This is similar to the phenotype of the *BUR2* deletion strain (76). The growth rates of the E107Q and D213N mutant strains were the same as that of *bur1*Δ, indicating that kinase activity is essential for Bur1 activity. The other alleles tested supported growth and were further assayed under a variety of conditions (Fig. 1D and E).

The *bur1-2* mutant grew slowly under all conditions. The C-terminal deletion (*bur1-C*Δ) and the point mutants T70A, T240A, and T240D grew similarly to wild-type cells at 30 or 37°C. The *bur1-C*Δ, T240A, and T240D strains are weakly cold sensitive (*cs* at 12°C but not 16°C). This suggests that both the C-terminal region and phosphorylation of T240 are necessary for full Bur1 activity. The new mutants did not share the sensitivity to caffeine (15 mM) or mild sensitivity to formamide (2%) displayed by the slower-growing *bur1-2* allele (Fig. 1D).

One possible effect of the mutants could be to weaken interactions between Bur1 and Bur2. If so, it might be possible to partially suppress the mutant phenotype by overexpression of Bur2. To test this possibility, *bur1* mutant strains were transformed with either a high-copy-number *BUR2* plasmid or empty vector, and the *cs* phenotype was reexamined. Overexpression of Bur2 partially suppresses the *cs* phenotypes of the T240A and T240D strains but not of the *bur1-2* or *bur1-C*Δ strains (Fig. 1F). This suggests that T-loop phosphorylation of Bur1 promotes interaction with Bur2, similar to the Kin28/Ccl1 interaction (34).

The *bur1-2* mutant allele was originally identified by increased in vivo transcription from a *SUC2* basal promoter with a deleted UAS (*suc2*ΔUAS). The new *bur1* mutants were tested for this ability in a modified *BUR1* shuffling strain (YSB799) that contains the *suc2*ΔUAS (−1900 to −390) locus (Table 1). The same strain can be used to determine the Spt phenotype, as it also contains the *lys2-128*δ locus in which *LYS2* transcription is disrupted by a Ty long terminal repeat sequence. As previously described, the *bur1-2* allele supports transcription from both these loci (57). Both the T240A and T240D alleles exhibit Bur$^-$ and Spt$^-$ phenotypes even stronger than *bur1-2*, perhaps due to their better overall growth rate. Although it shares the *cs* phenotype, the *bur1-C*Δ mutation is Spt$^+$ and only weakly Bur$^-$ (Fig. 1E). Overexpression of Bur2

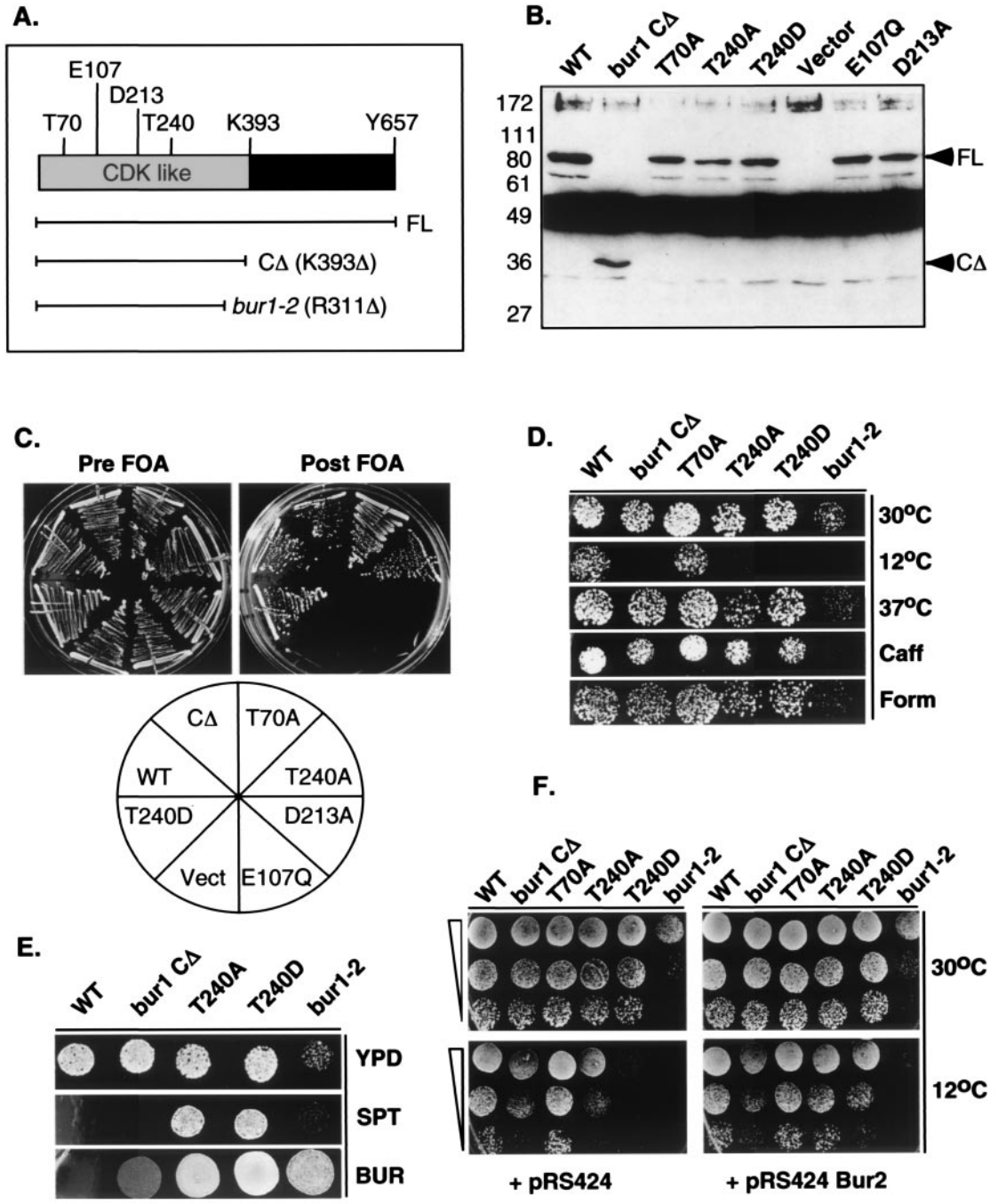

FIG. 1. In vivo analysis of Bur1 mutants. (A) Schematic of the Bur1 protein showing the residues targeted for mutation. (B) The mutant proteins are expressed at normal levels. The indicated HA_3-tagged *BUR1* alleles were used to replace the wild-type gene by plasmid shuffling. Whole-cell extracts were resolved by SDS-PAGE, and the expression levels were assayed by immunoblotting with anti-HA (12CA5) antibody. The very dark band near 50 kDa is a protein that cross-reacts with the 12CA5 antibody. FL, the full-length protein; CΔ, the truncation at amino acid 393; Vect, the vector plasmid with no *BUR1* gene. (C) Plasmid shuffling was performed on synthetic complete (SC) medium containing 5-FOA. All mutants except E107Q and D213A were able to support growth. (D) Cold-sensitive phenotypes of Bur1 T-loop and CTD mutants. Strains from panel C were spotted on various media for further analysis at the indicated temperatures. Growth at 30, 12, and 37°C was on yeast extract-peptone-dextrose (YPD); Caff, SC plus 15 mM caffeine; Form, YPD plus 2% formamide. (E) Spt and Bur phenotypes of mutants. Strains from panel C were assayed for Spt and Bur phenotypes. Spt^-, suppression of *lys2-128δ* allowing growth on SC-lysine; Bur^-, ability to bypass *suc2*ΔUAS (−1900 to −390) and support growth on 2% sucrose (+1 μg of actinomycin A/ml) as sole carbon source. (F) Overexpression of Bur2 partially suppresses the cold-sensitive (*cs*) phenotype of the T240A and T240D alleles. Strains were derived by plasmid shuffling and further transformed with high-copy-number vector (pRS424) or the same plasmid carrying the *BUR2* gene. Strains were spotted onto YPD and grown at 12 or 30°C.

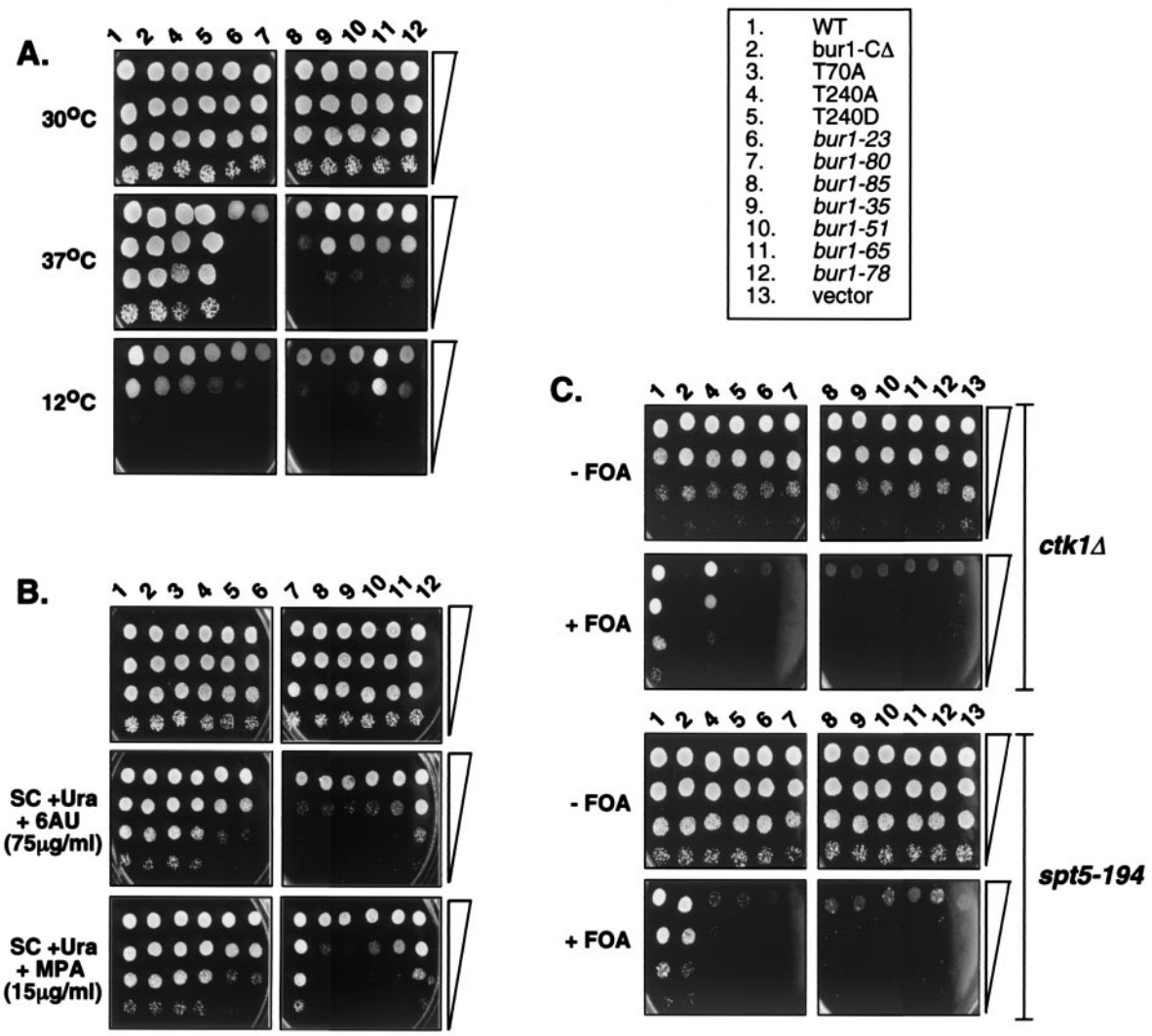

FIG. 2. Phenotypic analysis of Bur1 alleles. (A) Temperature-sensitive alleles of *bur1*. Mutants were introduced by plasmid shuffling and then spotted onto yeast extract-peptone-dextrose (10-fold dilutions between each row). Plates were incubated at 30°C (2 days), 37°C (2 days), and 12°C (5 days). (B) Some Bur1 mutants are sensitive to 6AU (75 μg/ml) and MPA (15 μg/ml). Strains shown in panel A were further transformed with pRS316 (URA3$^+$) and spotted onto synthetic complete medium (SC) plates with the indicated additions. (C) *BUR1* mutant alleles display synthetic genetic interactions with *ctk1*Δ and *spt5-194*. Double mutant strains were created by mating and transformation as described elsewhere (Table 1). Cells were spotted onto SC plates ± 5-FOA to remove the wild-type *BUR1* plasmid and grown for 2 days at 30°C.

has no effect on any of the Bur or Spt phenotypes (data not shown).

Since cold sensitivity is a difficult conditional phenotype to work with, a series of *bur1* temperature-sensitive (*ts*) alleles were isolated using random PCR-mediated mutagenesis and plasmid shuffling. Sequencing of the mutant ORFs shows each to contain multiple mutations (see Materials and Methods). At the nonpermissive temperature (37°C), the different alleles showed varying degrees of "tightness" (Fig. 2A). Additionally, most of the mutants grew slower than wild type at lower temperatures. The *bur1-C*Δ and T240 mutant alleles are not sensitive to high temperatures. The *bur1-23* strain was one of the tightest *ts* alleles identified and was therefore used in many of the experiments below. When tested for in vitro kinase activity, immunoprecipitated Bur1-23 protein had severely reduced activity relative to wild-type protein (data not shown). This was true whether yeast cells were grown at the permissive or nonpermissive temperature.

Bur1 and transcription elongation-related phenotypes. The *bur1-2* mutant is sensitive to the nucleotide analog 6AU and exhibits genetic interactions with mutant alleles of the elongation factors TFIIS (*PPR2*), *FCP1*, *CTK1*, and *SPT5* (44, 48). We examined the sensitivity of the defined *bur1* point mutants and *ts* alleles to the drugs 6AU and MPA. These chemicals depress cellular GTP levels, which is thought to affect the in vivo transcription elongation rate (18, 65). Mutations in any of several transcription elongation factors, which presumably further slow elongation, confer sensitivity to these drugs (4, 18, 43, 69).

The Bur1 C-terminal truncation strain was not sensitive to 6AU or MPA relative to wild type. The T-loop mutant T240D displayed mild sensitivity to 6AU (75 μg/ml) and MPA (15

μg/ml), while T240A did not. This parallels the greater cold sensitivity of T240D (Fig. 1F). The *ts* mutants all showed some 6AU and MPA sensitivity (Fig. 2B). One interesting exception was *bur1-80*, which is sensitive to 6AU but not MPA. For all the *bur1* alleles, sensitivity to these drugs was significantly less than that observed with a TFIIS deletion but comparable to deletion or mutation of other defined elongation factors such as *spt5-194, ctk1*Δ, and *rtf1*Δ (data not shown).

To test for genetic interactions between the mutant *bur1* alleles and known elongation factors, a series of strains were created containing *spt5-194*, *ctk1*Δ, or *ppr2*Δ in combination with *bur1*Δ::HIS3 covered by a *URA3*-marked *BUR1* plasmid (Table 1). The indicated, epitope-tagged Bur1 mutants were then introduced by plasmid shuffling. Although *bur1-2* has previously been reported as showing synthetic slow growth and temperature sensitivity in combination with *ppr2*Δ (48), we did not observe synthetic phenotypes between *ppr2*Δ and any of our new Bur1 mutants (data not shown). Since *bur1-2* results in an overall slow growth phenotype (Fig. 1D), it may be more sensitive to additional mutations. All the mutants were synthetic lethal with *ctk1*Δ, with the exception of T240A. In contrast, all mutants with the exception of *bur1-C*Δ were synthetic lethal with *spt5-194*, which grows slower than wild-type *BUR1* in this background (Fig. 2C).

Kinase activity of Bur1 mutant alleles. Bur1 can phosphorylate both the Rpb1 CTD and itself in vitro (48, 76). The catalytic activities of the conditional *Bur1-C*Δ, T240A, and T240D mutants were assayed on these substrates. Also analyzed were the E107Q and D213A mutant proteins that do not support growth (Fig. 1C). All mutants were expressed in the presence of untagged wild-type Bur1 so that there were no differences in growth rates. Bur1-containing complexes were immunoprecipitated from extracts via the epitope tag and then used in in vitro kinase assays. An HA-CTD fusion protein was used as substrate.

As expected, E107Q and D213A proteins are catalytically inactive. Unlike wild-type Bur1, the T240A and T240D mutants do not autophosphorylate, demonstrating that T240 is the site of T-loop phosphorylation. Furthermore, the T240 mutants have dramatically reduced activity towards the CTD (Fig. 3 and data not shown). In contrast, the *bur1-C*Δ allele is fully active for both autophosphorylation and CTD phosphorylation. From among the new temperature-sensitive alleles we generated, we tested the Bur1-23 protein and found that it was also severely decreased in kinase activity (data not shown). Thus, Bur1 mutants with reduced kinase activity can support yeast viability but lead to mutant phenotypes, while complete loss of Bur1 kinase activity is lethal.

To compare Bur1 to known physiological CTD kinases, Kin28, Srb10, and Ctk1 were immunoprecipitated and assayed in parallel. Efficient immunoprecipitation of all four kinases was confirmed by immunoblotting (Fig. 4A). All four kinases were active on recombinant GST-HA-CTD, although Bur1 produced the least phosphorylation of the group (Fig. 4B). CTD heptapeptide site preference of the kinase was examined by immunoblotting with the monoclonal antibodies 8WG16, H5, and H14. These primarily recognize CTD unphosphorylated at S2, S2-phosphorylated CTD, and S5-phosphorylated CTD, respectively (11, 53). Both Ctk1 and Srb10 showed a marked preference for S2, in agreement with previous reports

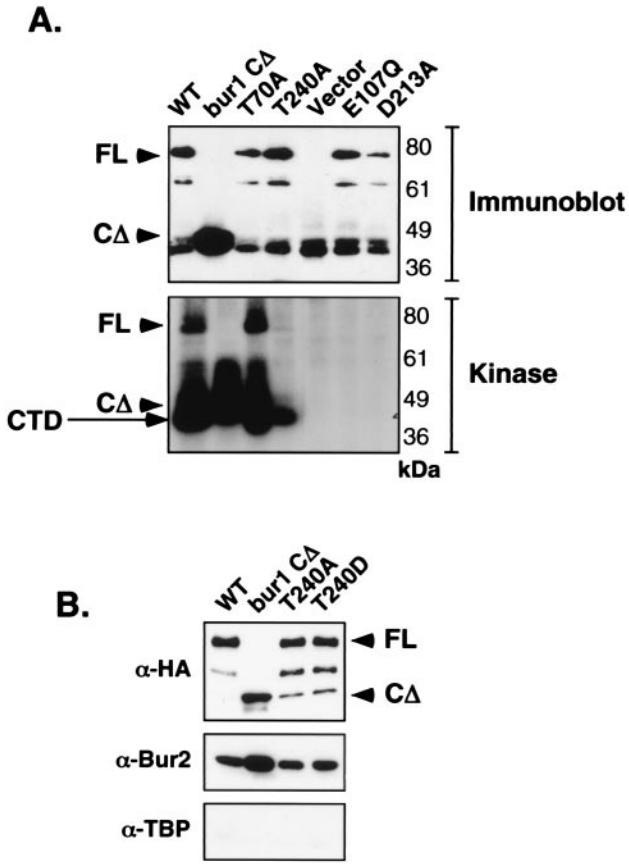

FIG. 3. In vitro CTD kinase activity of Bur1 mutant alleles. (A) Plasmids expressing epitope-tagged Bur1 mutants were transformed into YSB787 containing an untagged wild-type Bur1. Note that plasmid shuffling was not performed here, since the E107Q and D213A alleles are unable to support growth. Tagged Bur1-containing complexes were immunoprecipitated with 12CA5-protein A beads and split. In the upper panel, the precipitates were immunoblotted with anti-HA (12CA5) antibody. FL, the full-length protein; CΔ, the C-terminal truncation protein. Note that the immunoglobulin heavy chain migrates very closely to the Bur1 CΔ mutant, giving a background band. The other aliquots of the immunoprecipitates were tested for kinase activity with radiolabeled ATP and an HA-CTD fusion protein (see Materials and Methods). The lower panel shows SDS-PAGE of the reaction mixture exposed for autoradiography, showing both CTD phosphorylation and autophosphorylation of Bur1. Note that the Bur1 CΔ protein runs near the CTD substrate. Bur1 CΔ protein autophosphorylation was confirmed in reactions lacking the CTD substrate. (B) Immunoprecipitates were prepared as described for panel A. Blots were probed with anti-HA (12CA5) antibody to identify Bur1, anti-Bur2 (generously provided by Greg Prelich), or anti-TBP as a negative control.

(11, 26). In contrast, Kin28 phosphorylation was strongest at S5, as expected from its in vivo behavior (11, 12, 62, 64), although some H5 reactivity was also observed. Bur1 could weakly phosphorylate both S2 and S5 (Fig. 4B), although there was some preference for S5 as previously reported (48). It should be noted that our assays were done with GST-HA-CTD rather than the intact polymerase used in that previous study, which may explain the difference in the results.

Since mammalian cdk9 (P-TEFb) has been reported to phosphorylate TFIIF and Spt5 in vitro (35, 40, 58), we tested the ability of the immunoprecipitated kinases to phosphorylate

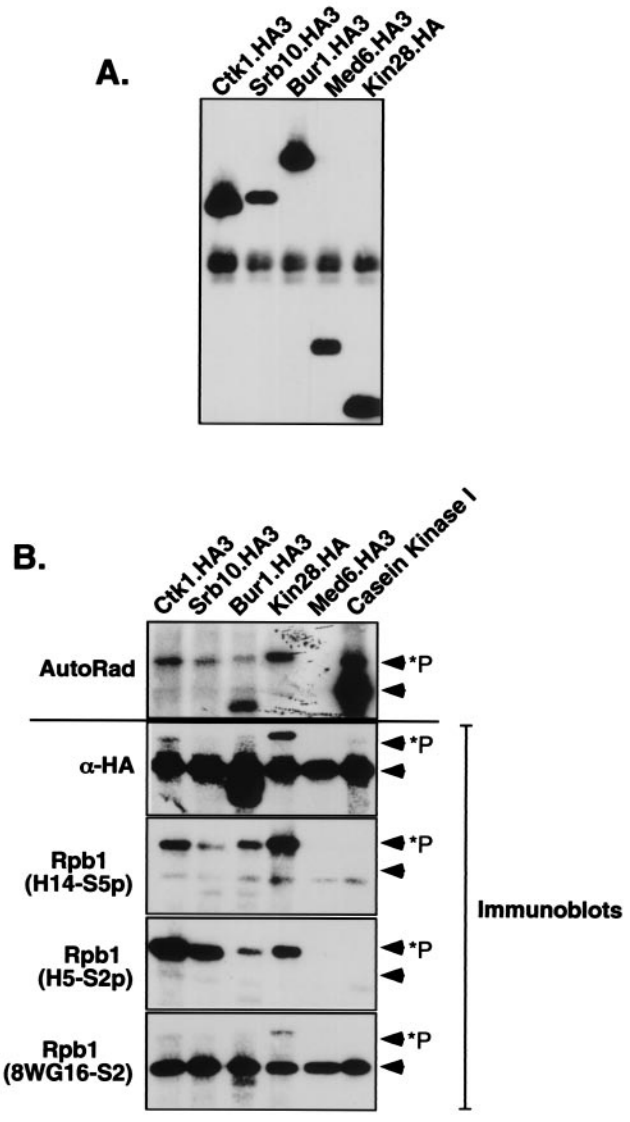

FIG. 4. Comparison of different CTD kinases. (A) Whole-cell extracts were prepared from the appropriate HA-tagged strains: Kin28 (YSB759), Ctk1 (YSB772), Srb10 (YSB776), Med6 (YSB777), and Bur1 (YSB770). The indicated epitope-tagged kinase complexes were immunoprecipitated with 12CA5-protein A beads and assayed by SDS-PAGE and immunoblot analysis using 12CA5. The common band in the middle is the antibody. (B) Kinase activity of immunoprecipitated protein complexes was assayed with [γ-^{32}P]ATP and recombinant GST-HA-CTD. Casein kinase I was used as a control. Phosphorylation of the CTD decreases its gel mobility shift (arrows with P* signify the phosphorylated forms). Different forms of GST-HA-CTD were visualized by autoradiography (top panel), 12CA5 antibody (anti-HA), or the phosphoepitope-specific monoclonal antibodies 8WG16 (anti-CTD), H5 (anti-CTD S2p), and H14 (anti-CTD S5p). The additional band below the unphosphorylated GST-HA-CTD in the Bur1 AutoRad and anti-HA panels is autophosphorylated HA-Bur1.

the corresponding yeast factors. No activity towards purified TFIIF (Tfg1, Tfg2, and Tfg3) was observed, while all four kinases were able to phosphorylate immunoprecipitated Spt5 complexes to the same extent (data not shown).

Bur1 and Bur2 are recruited to transcription complexes in vivo. Previous work (48) and the results above suggest a role for Bur1 in elongation. If so, Bur1 could be associated with transcribing complexes in vivo. This was tested using ChIP (51, 52). Epitope-tagged Bur1 cross-links near the promoter and throughout the coding sequence of the constitutively transcribed *PMA1* gene but not to a nontranscribed intergenic region of chromosome V (Fig. 5). Bur2, the cyclin partner of Bur1, shows exactly the same pattern. Similar results were obtained with the *ADH1*, *DBP2*, *PDR5*, *PYK1*, and *YEF3* genes (Fig. 6 and data not shown). ChIP analysis of galactose-inducible genes showed that Bur1 is not associated when these genes are repressed by growth in glucose-containing medium. Similarly, the TATA binding protein (TBP) is not at the *GAL* promoters in the absence of transcription. Both TBP and Bur1 are strongly cross-linked after induction with galactose (Fig. 5B). Thus, Bur1 recruitment to genes correlates with transcription. Based on empirical observations, the resolution of the ChIP assay is about 200 to 300 bp. Therefore, we cannot use this assay to conclude whether Bur1/Bur2 is associated with preinitiation complexes or early elongation complexes. However, a recent study of preinitiation complexes by quantitative mass spectroscopy did not detect either Bur1 or Bur2 (61).

Surprisingly, cross-linking of Bur1 and Bur2 to the *PMA1* gene was lower downstream of the ORF relative to RNApII (Fig. 5A). The last *PMA1* primer pair is downstream of the polyadenylation site (M. Kim and S. Buratowski, unpublished data). To test whether this is a general phenomenon, we compared the cross-linking of Bur1 to that of Rpb3 on the genes *PMA1*, *ADH1*, *YEF3*, and *PYK1* (Fig. 6). At each gene, the ratio of Bur1 to Rpb3 was similar at the promoter and coding sequence, but Bur1 levels were reduced after the poly(A) site. Since RNApII transcribes past the poly(A) site (16), these results raise the possibility that the termination and polyadenylation signals trigger release of Bur1/2 from the elongation complex. The drop in Bur1 levels was 20 to 50%, depending on the gene tested, perhaps reflecting different termination efficiencies or placements of the primers relative to the polyadenylation site (7, 8, 17).

We tested whether Bur1 cross-linking was affected in the mutant strains. Epitope-tagged mutant Bur1 proteins were assayed in the presence of the endogenous, untagged wild-type allele. This ensured that growth rates of strains were similar and showed whether the mutant alleles can compete for occupancy with the wild-type allele. Despite their differences in catalytic activities, the Bur1 T240A, T240D, and E107Q mutants showed the same pattern and intensity of cross-linking as wild type (Fig. 7A). The finding that catalytically inactive Bur1 can still associate with elongation complexes may suggest that Bur1 can exchange or that multiple Bur1 molecules are present during elongation. The Bur1-CΔ protein consistently showed reduced cross-linking ($\approx$50% of wild type). This suggests either that the C-terminal half of Bur1 is important for association with the elongation complex or that the truncation simply reduces the cross-linking efficiency of the protein. We also examined cross-linking of the T240A T-loop mutant protein in the absence of wild-type protein. Bur1(T240A) cross-linked identically to wild-type protein, including the drop downstream of the polyadenylation site (Fig. 7B). Therefore, Bur1 association with the elongating polymerase does not require kinase activity or T-loop phosphorylation.

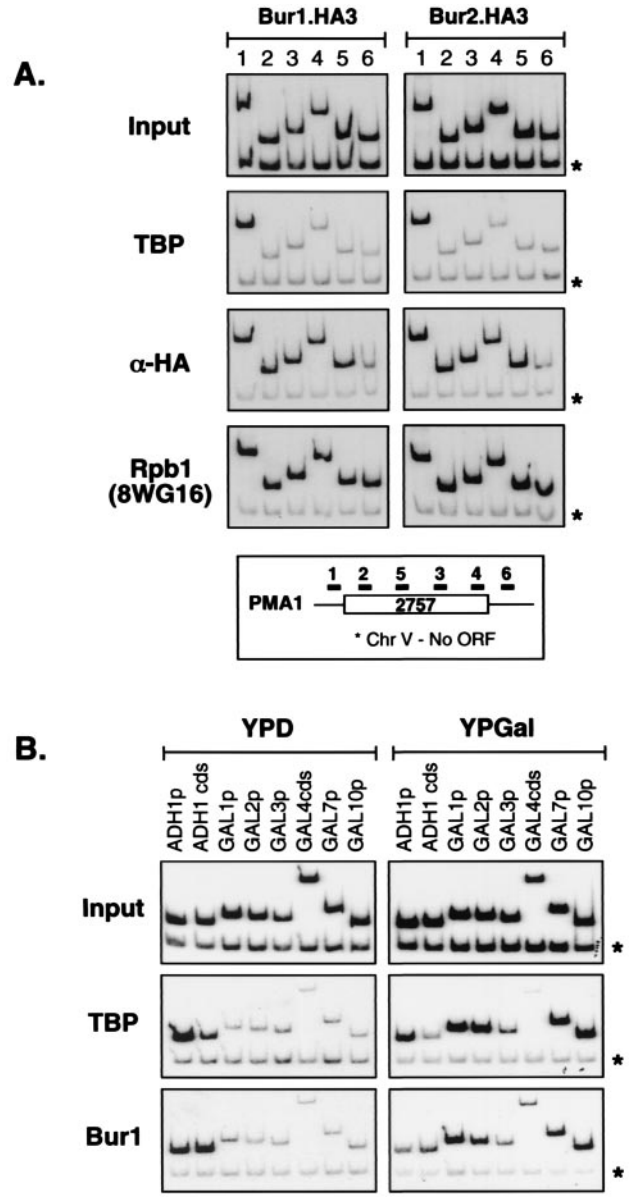

FIG. 5. Bur1 is associated with transcription elongation complexes in vivo. (A) HA_3-tagged Bur1 (YSB770) and Bur2 (YSB813) strains were analyzed using ChIPs as described in Materials and Methods. Locations of the PMA1 primers for PCR are depicted schematically. The number within the rectangle gives the size of the ORF in base pairs. The upper panel is the input used to normalize the PCR amplification. Samples were immunoprecipitated with anti-TBP, monoclonal antibody 12CA5 recognizing the HA-tagged Bur1 (left column) or Bur2 (right column), or anti-CTD (8WG16). The asterisk marks a nontranscribed PCR fragment included in all reactions as a background control. (B) ChIPs were carried out on several galactose-inducible genes to examine de novo recruitment of transcription complexes. Cells were grown in 2% raffinose and then switched to 2% glucose (GLU) or 2% galactose (GAL) for 4 h before formaldehyde cross-linking and ChIP analysis. Immunoprecipitations were done with anti-TBP and anti-HA antibodies recognizing the tagged Bur1 protein. Primers amplified the constitutive ADH1 promoter (Adh1p) or coding sequence (cds) or the corresponding locations within the indicated GAL genes.

Effect of Bur1 mutants on transcription elongation complexes. To determine whether Bur1 activity is important for transcription elongation, ChIP of RNApII was carried out in wild-type and *bur1-23* strains. We have shown that Bur1-23 has significantly reduced in vitro kinase activity (data not shown). Strains were grown at room temperature (25°C) and shifted to 37°C for 2 and 4 h. As a positive control for elongation defects, cross-linking was also carried out in the presence of 25 μg of 6AU/ml (Fig. 8A). This dose was chosen as the lowest tested (of 10, 25, 50, or 75 μg/ml) in spot analyses at which a panel of elongation factor mutants (*ppr2*Δ, *ctk1*Δ, *spt5-194*, and *bur1-23*) showed a selective sensitivity to 6AU relative to wild-type cells (data not shown).

In a wild-type strain, RNApII cross-linked to promoters and coding regions at both temperatures. In the presence of 6AU, both Rpb1 and Rpb3 showed reduced cross-linking that became more pronounced with distance from the promoter (Fig. 8A). In contrast, recruitment of polymerase (Rpb1 and Rpb3) and TBP to the promoter region was relatively unaffected following 6AU treatment. The simplest interpretation of this pattern is that most polymerases initiating transcription at the promoter are unable to reach the 3′ end of the gene.

At the permissive temperature, the *bur1-23* strain exhibited a pattern of RNApII cross-linking slightly different from that of the wild-type strain. A slight reduction in cross-linking was noted with primers further away from the transcription start site. Growth of the *bur1-23* strain for 2 or 4 h at the nonpermissive temperature (37°C) preferentially reduced the cross-linking of Rpb1 or Rpb3 at the 3′ end of *PMA1* relative to that of the promoter (Fig. 8B and C). This polarity was similar to that seen in the presence of 6AU, suggesting that loss of Bur1 activity causes a decrease in elongation efficiency. This effect is not allele specific, because a similar pattern was observed with the *bur1-80* strain (data not shown). We also monitored the presence of the mutant Bur1-23 protein (using the HA tag). The mutant Bur1 pattern paralleled that of Rpb3 at all temperatures (data not shown), indicating that the mutant protein remains associated with the elongation complex.

In order to determine whether Bur1 contributes to CTD S5 or S2 phosphorylation during transcription, cross-linked chromatin was immunoprecipitated with the monoclonal antibodies H14 and H5 (36) (Fig. 8B). The H14 (S5 phosphorylation) signal was relatively unaffected in the *bur1-23* or *bur1-80* mutants (Fig. 8B and C and data not shown). Also, the H5 signal (S2 phosphorylation) remained easily detectable even as the total amount of polymerase II cross-linking was decreased by 6AU or the *bur1-23* mutant. Since there are 27 copies of the H5 epitope within the CTD, it is not surprising that its detection limit will be lower than that of Rpb3. When the H5 and H14 signals were normalized to the Rpb3 signal, no relative decrease in CTD phosphorylation was observed. These results cannot rule out the possibilities that Bur1 contributes to a minor proportion of CTD phosphorylation or phosphorylates the CTD in a pattern that is not recognized by the H5 and H14 antibodies. However, the ChIP results suggest that the majority of cotranscriptional CTD phosphorylation at both S5 and S2 is normal in the absence of Bur1 activity.

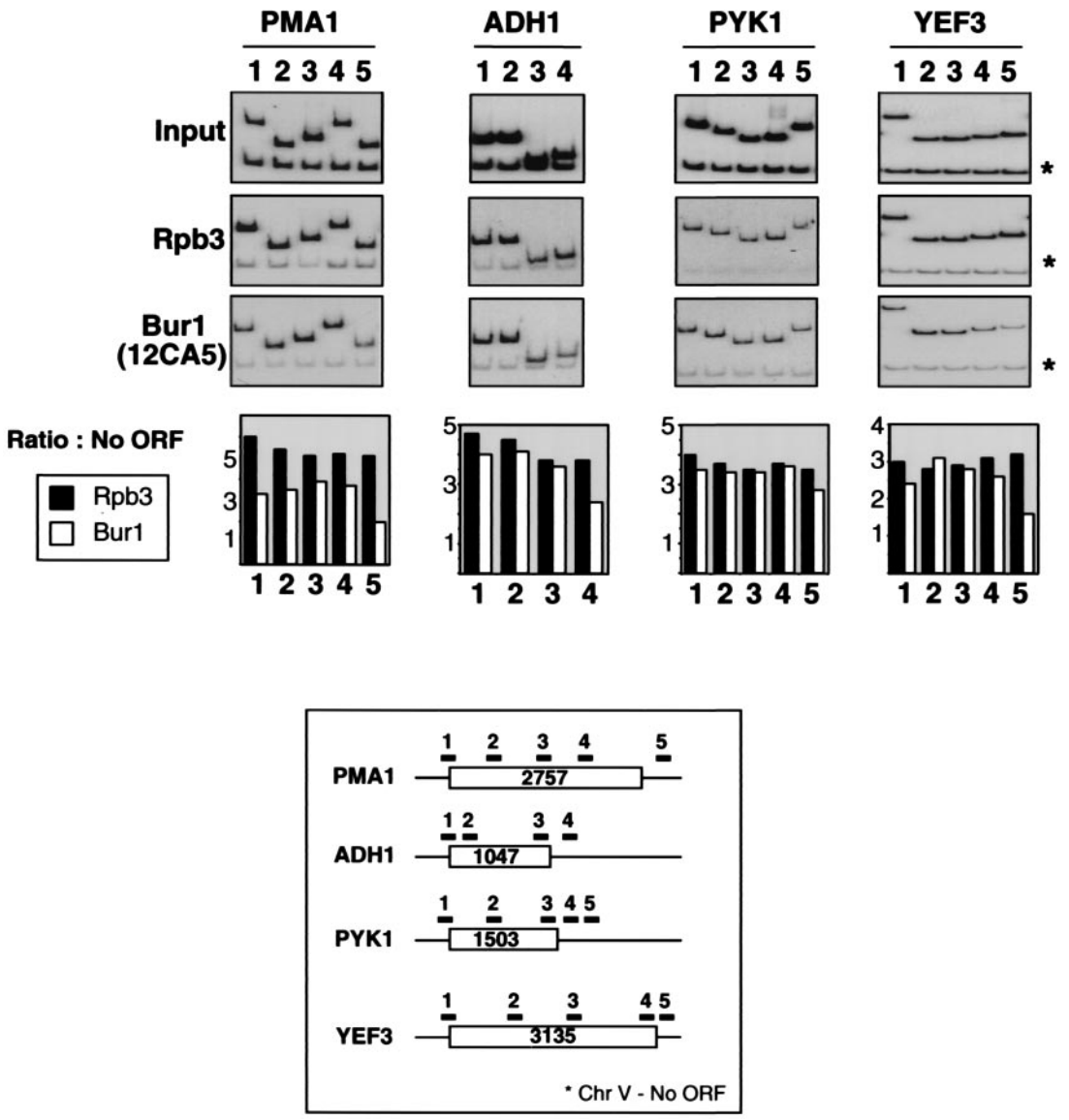

FIG. 6. Bur1 cross-linking is reduced at the 3′ end of genes. ChIPs were performed with an HA_3-tagged Bur1 strain (YSB770). Immunoprecipitation was carried out with anti-Rpb3 (a polymerase II subunit) and anti-HA recognizing the tagged Bur1. Various regions of the constitutively expressed genes *PMA1*, *ADH1*, *PYK1*, and *YEF3* were examined (locations of the primer pairs are depicted in the bottom panel). Quantitation of the PCR results after normalization to input is graphed below the gels. The numbers (*y* axis) designate the signal of the specific primer pair product relative to the nontranscribed no-ORF negative internal control.

DISCUSSION

The Ctk1 kinase associates with elongation complexes (11, 33, 42). It is required for cotranscriptional phosphorylation of the Rpb1 CTD on S2, with phosphorylation levels increasing as elongation proceeds through the gene (11). Our ChIP results show that the Bur1 kinase and its associated cyclin are also present in elongation complexes. Bur1 mutants display a number of phenotypes that suggest a role in elongation, but ChIP experiments using Bur1 conditional mutants provide the clearest evidence yet. In Bur1 mutant cells, RNApII assembles efficiently at promoters, but most complexes fail to reach the 3′ end of genes (Fig. 8). A similar polarity is seen upon treating cells with 6AU but, surprisingly, not in a Ctk1 deletion strain (11). Despite the fact that elongation is clearly affected in the Bur1 mutants, CTD phosphorylation patterns during transcription appear normal. Bur1 mutant alleles and *ctk1*Δ share many genetic interactions. Mutants in both genes display sensitivity to 6AU and MPA (Fig. 2B) (33, 48, 65, 73) and synthetic lethality with *spt5-194*, *spt4*Δ, and *kin28-16* (44) (Fig. 2). Despite these similarities, *BUR1* and *CTK1* are unable to functionally substitute even when overexpressed (data not shown) (77). Spt^- and Bur^- phenotypes (Fig. 1) are seen with Bur1 but not Ctk1 mutants. Together, the ChIP and genetic data indicate that Ctk1 and Bur1 have nonoverlapping roles in transcription.

Bur1 has sequence motifs of a typical CDK. Mutants with substitutions at important catalytic residues are not functional in vivo (Fig. 1C). In agreement with Yao and Prelich (77), we

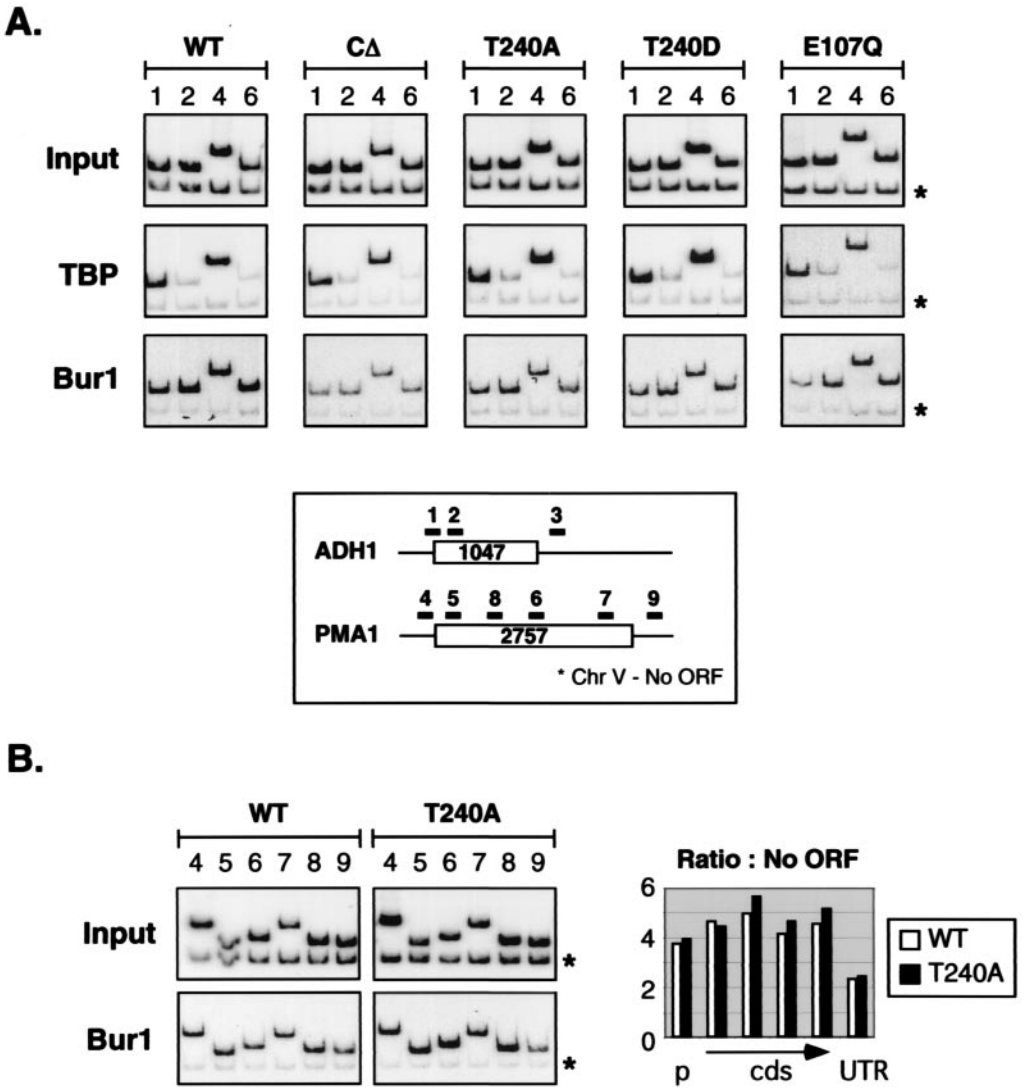

FIG. 7. Bur1 mutants do not affect association with transcription complexes. (A) Epitope-tagged Bur1 mutant alleles were transformed into strain YSB787, and the resulting strains were used for ChIP analysis in the presence of untagged wild-type Bur1. Cross-linking of each mutant to promoter and coding sequences of the *PMA1* and *ADH1* genes is shown. (B) The Bur1 T240A allele shows a similar cross-linking pattern to wild-type Bur1 throughout the *PMA1* gene, indicating that phosphorylation of the Bur1 T-loop is not the signal for disengagement at the 3′ untranslated region (UTR). Note that the T240 mutant strain does not contain wild-type Bur1.

found that substitutions at the T-loop phosphorylation site are viable but with several mutant phenotypes (Fig. 1 and 2). The T-loop mutant proteins have reduced kinase activity, although this does not appear to be caused by less-efficient association with Bur2 (Fig. 3). Both catalytically inactive and T-loop mutants associate with elongation complexes, indicating that Bur1 kinase activity is not required for the association (Fig. 7).

In addition to the N-terminal CDK domain, Bur1 has an additional C-terminal region. This domain is partially conserved in yeast species of the *Saccharomyces* genus, but it is quite divergent even in other yeasts, such as *Schizosaccharomyces pombe* and *Candida albicans* (P. Cliften and M. Johnston, Washington University Genome Sequencing Center, personal communication). There is no sequence similarity of this region to other nonhomologous proteins in the sequence databases. Deletion shows that the C-terminal region is not essential for viability or kinase activity, but it does lead to a cold-sensitive phenotype. Cold sensitivity was also observed when a C-terminal truncation of the *S. pombe* Bur1 homologue was used to replace the *S. cerevisiae* gene (54). The Bur1 C-terminal region may contribute to recruitment of Bur1 to elongation complexes (Fig. 7) or interact with other components of the transcription complex, such as the capping enzyme triphosphatase (54).

What is the molecular function of Bur1? The Bur1/2 complex is recruited to genes coincident with transcription (Fig. 5). However, the resolution of the ChIP technique does not allow us to distinguish whether it is part of the preinitiation or early elongation complex. The inefficiency of elongation complexes in reaching the 3′ end of genes seen in *bur1* temperature-sensitive strains clearly indicates a role in promoting elongation (Fig. 8B and C). This defect is most notable on long genes

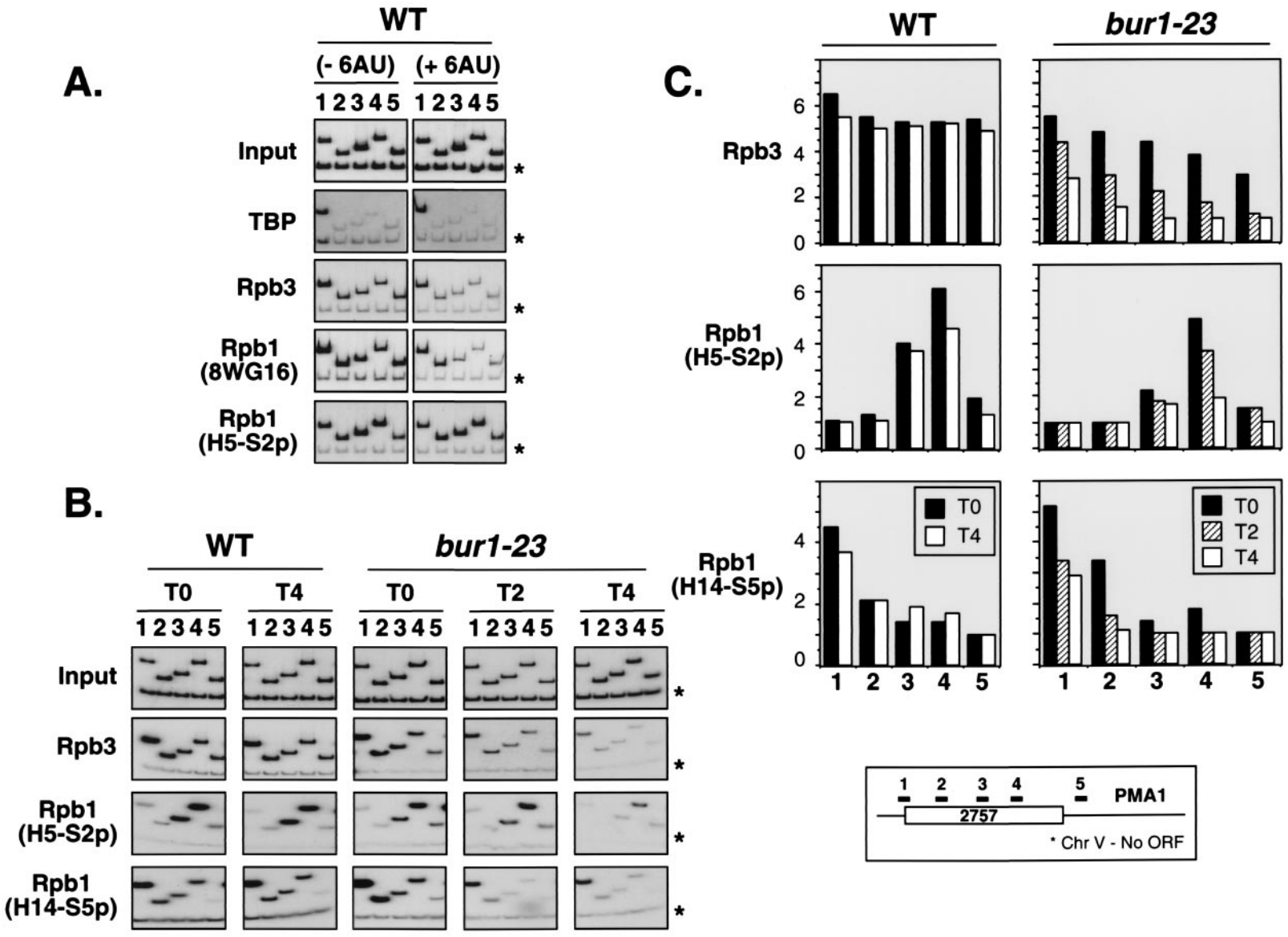

FIG. 8. Bur1 is required for transcription elongation but not cotranscriptional CTD phosphorylation. (A) An elongation block by 6AU leads to a 5′-to-3′ polarity in cross-linking of RNApII subunits Rpb1 and Rpb3. Cells were grown for 4 h in the presence of 6AU at 25 μg/ml, followed by formaldehyde cross-linking and ChIP. Although distal levels of Rpb1 and Rpb3 decrease in the presence of 6AU, note that S2 phosphorylation remains robust. (B) Wild-type or *bur1-23* cultures were grown at 25°C and shifted to the nonpermissive temperature for 2 h (T2) and 4 h (T4). Formaldehyde cross-linking and ChIP were performed as previously described. Total levels of RNApII were assayed with anti-Rpb3, while CTD phosphorylations at S2 and S5 were tested with the indicated monoclonal antibodies. (C) The results in panel B were quantitated by phosphorimager analysis and graphed. Signals from specific primer products (diagrammed at bottom) were normalized to input and quantitated relative to the nontranscribed no-ORF internal control.

(data not shown) and resembles the effect seen with 6AU, a drug that promotes transcriptional stalling by lowering GTP concentrations. The effects of *bur1* mutants and 6AU on elongation are additive (data not shown), explaining the 6AU sensitivity of many *bur1* mutant alleles. Surprisingly, Bur1/2 crosslinking downstream of polyadenylation sites is significantly reduced relative to Rpb1 and Rpb3 (Fig. 6). The signal for this apparent dissociation does not involve T-loop phosphorylation of Bur1. Dissociation of a kinase that promotes elongation could be part of the transcription termination mechanism.

Presumably, Bur1 phosphorylates some substrate to either prevent termination or promote elongation. Based on its coimmunoprecipitation of RNApII with Bur1, it has been suggested that the substrate is S5 of the Rpb1 CTD (48). The CTD can act as a Bur1 substrate in vitro (Fig. 4) (48), although we find that Bur1 has lower activity and less site preference than three previously characterized CTD kinases (Kin28, Ctk1, and Srb10). In our ChIP experiments, phosphorylation of CTD S2 or S5 appeared normal in the *bur1-23* or *bur1-80* temperature-sensitive mutants, even at the nonpermissive temperature when the effect on elongation was quite pronounced (Fig. 8 and data not shown). Furthermore, on immunoblots of whole-cell extracts from a *bur2*Δ strain, levels of S5 phosphorylation (as assayed with the monoclonal antibody H14) are normal (M. Kim and S. Buratowski, unpublished data). Although there is a slight reduction of S2 phosphorylation (i.e., H5 reactivity) in these extracts, this may be an indirect effect caused by the lower levels of elongating polymerase. In this context, it is interesting to note that CTD truncation or substitution mutants have no 6AU or MPA sensitivity (data not shown). For all these reasons, we suspect that the important Bur1 substrate is something other than the RNApII CTD.

Clues to the substrate of Bur1 may come from higher eukaryotes. Both Bur1 and Ctk1 are equally similar in sequence to higher eukaryotic Cdk9, so an exact homologue relationship has not yet been established. Cdk9 and cyclin T form a complex known as P-TEFb that stimulates elongation in vitro (31, 56). P-TEFb is proposed to function by phosphorylating the CTD during elongation, which serves to prevent polymerase arrest (45, 58). The exact heptapeptide specificity of Cdk9 is controversial. Using peptides, Ramanathan et al. found a preference for S5 that is inhibited by prior phosphorylation of S2 (60). Zhou et al. have reported that Cdk9 phosphorylates S2 within initiation complexes in vitro, but this substrate specificity is altered in the presence of the human immunodeficiency virus transactivator protein Tat. The resulting complex phosphorylates both S2 and S5 (78). Using RNA interference to knock down Cdk9 in *Caenorhabditis elegans* embryos, Shim et al. found a loss of S2 but not S5 phosphorylation (66).

The function of Cdk9 may also be mediated via another substrate, the elongation factor Spt5 (35, 55, 66, 74). Spt5 was originally identified and implicated in transcription by a yeast genetic screen (75). The Spt4/5 complex associates with RNApII and plays a role in transcription elongation in vivo (25, 37, 69), possibly by altering chromatin structure in combination with Spt6 (9). The human Spt4/5 complex is known as DSIF and plays a role in elongation (10, 74). It has been proposed that phosphorylation of Spt5 regulates DSIF activity, with the nonphosphorylated form acting as a repressor. Phosphorylation by P-TEFb may alleviate this repression (55, 66, 74).

One interesting idea is that *S. cerevisiae* Bur1 and Ctk1 are both paralogues of Cdk9, having arisen by divergent evolution from a common ancestor. Whereas Cdk9 may act upon both the CTD and Spt4/5, *S. cerevisiae* may utilize distinct CDK complexes for these two functions. Ctk1 appears to be dedicated to CTD S2 phosphorylation, while Bur1 might act upon Spt4/5. In support of this idea, mutants in *BUR1/2* and *SPT4/5/6* share a number of phenotypes (24, 49, 57, 71, 72). Partial loss-of-function *BUR1* alleles show synthetic phenotypes with *spt5-194* and *spt4*Δ, suggesting a common pathway (44) (Fig. 2C). We have found that Spt5 can act as a Bur1 substrate in vitro, although it is also phosphorylated by Kin28, Ctk1, and Srb10 (data not shown). A putative Bur1 homologue from *S. pombe* can also phosphorylate SpSpt5 in vitro (54). Spt5 contains several sequence motifs reminiscent of the CTD YSPT SPS heptapeptide. Further experiments will be required to determine whether these are physiological targets of Bur1 or Cdk9.

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