The Bur1 Cyclin-Dependent Protein Kinase Is Required for the Normal Pattern of Histone Methylation by Set2

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BUR1 and BUR2 encode the catalytic and regulatory subunits of a cyclin-dependent protein kinase complex that is essential for normal growth and has a general role in transcription elongation. To gain insight into its specific role in vivo, we identified mutations that reverse the severe growth defect of *bur1Δ* cells. This selection identified mutations in SET2, which encodes a histone methylase that targets lysine 36 of histone H3 and, like BUR1, has a poorly characterized role during transcription elongation. This genetic relationship indicates that SET2 activity is required for the growth defect observed in *bur1Δ* strains. This SET2-dependent growth inhibition occurs via methylation of histone H3 on lysine 36, since a methylation-defective allele of SET2 or a histone H3 K36R mutation also suppressed *bur1Δ*. We have explored the relationship between BUR1 and SET2 at the biochemical level and find that histone H3 is monomethylated, dimethylated, and trimethylated on lysine 36 in wild-type cells, but trimethylation is significantly reduced in *bur1* and *bur2* mutant strains. A similar methylation pattern is observed in RNA polymerase II C-terminal domain truncation mutants and in an *spt16* mutant strain. Chromatin immunoprecipitation assays reveal that the transcription-dependent increase in trimethylated K36 over open reading frames is significantly reduced in *bur2Δ* strains. These results establish links between a regulatory protein kinase and histone methylation and lead to a model in which the Bur1-Bur2 complex counteracts an inhibitory effect of Set2-dependent histone methylation.

Generation of a mature mRNA requires successful completion of a series of steps, including promoter recognition, assembly of a preinitiation complex, initiation of pre-mRNA synthesis, promoter clearance, elongation, termination, capping, 3'-end formation, and splicing. Each of these steps can have regulatory roles at subsets of genes, and therefore it is important to identify the global and gene-specific factors required for each of these steps and their mechanism of action.

In recent years a growing number of factors have been implicated in transcription elongation, either genetically or biochemically. Included among these are the Bur1-Bur2 cyclin-dependent kinase (Cdk) complex (59), Set2 (20, 47, 58), Spt16 (FACT subunit) (35), Spt6 (12), and the Spt4-Spt5 complex (also called DSIF) (12, 56). Analysis of mutations in the genes that encode suspected transcription elongation factors in *Saccharomyces cerevisiae*, both individually and in combination, suggests that they are important; deletion of *SPT16* (FACT subunit) (28), *SPT5* (DSIF subunit) (53), and *SPT6* (7) is lethal. Deletion of other elongation factor genes such as *PPR2* (TFIIS) (33), *SPT4* (DSIF subunit) (29), *CTK1* (24), or *SET2* (52) is not lethal, but this is at least partially due to functional redundancy, since combinatorial effects are frequently observed when these deletions are combined. The conservation of these factors from yeast to humans further attests to their biological importance, and their involvement in human diseases (8, 49) emphasizes the necessity for a greater understanding of their roles.

BUR1 (also known as *SGV1*) was originally identified in *S.*

cerevisiae as a protein kinase with an unspecified role in the recovery of yeast from mating pheromone-induced cell cycle arrest (13). Its role in the pheromone pathway has never been clarified beyond that original report, but a breakthrough in understanding this gene came with its discovery in a genetic selection for mutations that increase transcription from a promoter that lacks an upstream activating sequence (UAS) in yeast (42). The reporter gene for the selection, *SUC2*, is required for growth of yeast on sucrose-containing media; strains that contain the *suc2Δuas*(–1900/–390) allele do not transcribe *SUC2*, and are therefore unable to grow on sucrose plates (46). Selection for Suc⁺ revertants of *suc2Δuas*(–1900/–390) identified mutations in a subset of previously characterized *SPT* genes and six other genes, designated *BUR1* through *BUR6* (for bypass UAS requirement) (42).

All of the proteins identified using this selection have general roles in transcription, including histones and other chromatin-related regulators, TATA-binding protein (TBP), regulators of TBP, and elongation factors (4, 40, 42, 59). This selection therefore provided a solid genetic link between *BUR1* and transcription. In addition, mutations in a second gene identified by the Bur selection caused a spectrum of phenotypes virtually identical to that of *bur1* mutations, suggesting that it might serve as a regulator or substrate of the Bur1 kinase. Using a variety of genetic and biochemical approaches, we were able to show that this gene, *BUR2*, encodes a cyclin that is directly required for Bur1 kinase activity (59). The Bur1-Bur2 complex thus became the fourth cyclin-dependent protein kinase in yeast postulated to have general roles in transcription (39).

Several lines of evidence suggest that the Bur1-Bur2 complex has a role during transcription elongation. First, *bur1* and

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TABLE 1. Yeast strains

Strain	Relevant genotype
AY272	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 ura3-52 leu2Δ1 bur1Δ1::TRP1 mut3::LEU2</i>
AY288	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 ura3-52 leu2Δ1 bur1Δ1::TRP1 mut59::LEU2</i>
AY301	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 ura3-52 set2Δ::KAN</i>
AY311	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 ura3-52 leu2Δ1 set1Δ::KAN</i>
AY315	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 leu2Δ1 ura3-52 dot1Δ::KAN</i>
AY337	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 ura3-52 bur1Δ1::TRP1 set2Δ::KAN</i>
AY340	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 ura3-52 leu2Δ1 bur1Δ1::TRP1 set2Δ::KAN</i>
AY360	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) leu2Δ1 trp1Δ63 set2Δ::KAN bur2Δ3::TRP1</i>
AY403	<i>MATα his3Δ200 lys2-128δ ura3-52 trp1Δ901 leu2Δ1 bur1Δ1::KAN hht1-hhf1::LEU2 hht2-hhf2::HIS3 <CEN TRP1 hht2K36R-HHF2></i>
AY447	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) ura3-52 trp1Δ63 leu2Δ1 bur1Δ1::TRP1 <CEN LEU2 flag-bur1-8></i>
AY588	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 ura3-52 leu2Δ1 eaf3Δ::KAN</i>
AY658	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 ura3-52 chd1Δ::URA3</i>
AY664	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 ura3-52 leu2Δ1 bur1Δ1::TRP1 chd1Δ::URA3</i>
AY672	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 ura3-52 leu2Δ1 bur1Δ1::TRP1 eaf3Δ::URA3</i>
FY2139	<i>MATα lys2-128δ ura3-52 leu2Δ1 FLAG-spt6-1004</i>
GY80	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 ura3-52 spt5-194</i>
GY83	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) leu2Δ1 ura3-52 spt16-197</i>
GY193	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) ura3-52 trp1Δ63 bur2Δ2::URA3</i>
GY324	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63 spt6-140</i>
GY471	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) trp1Δ63</i>
GY480	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) leu2Δ1 trp1Δ63 ura3-52</i>
GY726	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) leu2Δ1 ura3-52 spt4Δ::URA3</i>
GY753	<i>MATα his3Δ200 lys2-128δ suc2Δuas(−1900/−390) ura3-52 ctk1Δ::HIS3</i>
GY828	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) ura3-52 trp1Δ63 bur2Δ3::TRP1</i>
OY56	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) his3Δ200 ura3-52 leu2 rpb1Δ187::HIS3 <rpb1Δ104 LEU2 CEN></i>
OY57	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) his3Δ200 ura3-52 leu2 rpb1Δ187::HIS3 <rpb1Δ103 LEU2 CEN></i>
OY58	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) his3Δ200 ura3-52 leu2 rpb1Δ187::HIS3 <rpb1Δ101 LEU2 CEN></i>
OY150	<i>MATα his3Δ200 lys2-801 trp1Δ901 ura3-52 leu2Δ1 ade2-101 hht1-hhf1::LEU2 hht2-hhf2::HIS3 <hht2K36R HHF2 TRP1></i>
OY221	<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rad6Δ::KAN</i>
RY19	<i>MATα his4-912δ lys2-128δ suc2ΔUAS(−1900/−390) ura3-52 trp1Δ63 leu2Δ1 bur1Δ1::TRP1 <CEN LEU2 flag-BUR1></i>
SY54	<i>MATα his4-912δ lys2-128δ suc2Δuas(−1900/−390) ura3-52 trp1Δ63 leu2Δ1 bur1Δ1::TRP1 <CEN URA3 BUR1></i>

bur2 mutations are synthetically lethal with mutations in *CTK1*, which encodes a protein kinase that phosphorylates RNA polymerase during elongation, and with mutations in *SPT4* and *SPT5*, which encode subunits of the DSIF elongation factor (31). In addition, synthetic sickness was observed in combination with *rpb1* and *rpb2* elongation-defective mutations and with mutations in the *SPT6* histone chaperone, TFIIS, and the *FCPI* C-terminal domain (CTD) phosphatase. The synthetic phenotypes were specific, as no combinatorial defects were observed when *bur1* mutations were combined with mutations in *KIN28*, *SRB10*, or the genes that encode the *Srb4* mediator subunit or histones H2A and H2B. Second, *bur1* and *bur2* strains are sensitive to 6-azauracil (31), a phenotype that is a frequent indicator of a transcription elongation defect. The conclusion that Bur1 is an elongation factor has been corroborated by recent chromatin immunoprecipitation results showing that Bur1 and Bur2 are recruited to open reading frames of transcribed genes in a transcription-dependent manner and that RNA polymerase II association with open reading frames is defective in *bur1* and *bur2* mutant strains (17).

Although the specific role of the Bur1-Bur2 Cdk and its relationship to other elongation factors remain unknown, the similarity of *bur1* phenotypes to *spt5*, *spt6*, and *spt16* phenotypes, including the ability of *bur1* mutations to suppress *snf5 Δ* and *snf5 Δ* (42), suggested that *bur1* affects transcription through a chromatin-mediated mechanism. To gain further insight into the role of Bur1-Bur2 in vivo, we selected mutations that suppress the severe growth defect caused by a *bur1* deletion. This selection revealed a new functional link between

the Bur1 kinase and Set2, a histone methylase that was independently implicated in transcription elongation.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* strains used in this study are listed in Table 1. All media, including yeast-peptone-dextrose (YPD), synthetic complete drop-out medium (e.g., SC-Ura), and minimal and sporulation media were made as described previously (44). 5-Fluoroorotic acid (5-FOA) sensitivity was assayed on media that contained 1 g/liter 5-FOA, and medium containing 2% formamide was used to assay formamide sensitivity. Standard genetic methods for mating, sporulation, transformation, and tetrad analysis were used throughout this study (44).

Genetic methods. A yeast genomic library containing mTn-*LEU2/lacZ* insertions, provided by P. Ross-Macdonald and M. Snyder (Yale University, New Haven, CT), was used to mutagenize SY54 (*bur1 Δ* [*pGP59* = *CEN URA3 BUR1*]) (see Table 1) essentially as described (3). Approximately 51,000 *Leu*⁺ transformants were obtained and replica plated to SC-Leu-5-FOA plates. 5-FOA-resistant colonies were selected after 3 to 4 days of growth and used as templates in PCRs using primers diagnostic for the *BUR1* open reading frame. These primers generate an 880-bp PCR product indicative of *BUR1*⁺ and a 1,937-bp PCR product indicative of *bur1 Δ* ::*TRP1*. 5-FOA-resistant candidates that generated only the 1,937-bp band were pursued further. Candidates were crossed with GY480 (*BUR1*⁺) and tetrads were dissected to determine if viability was linked to the transposon-based *LEU2* marker. Two transposon-linked suppressors tentatively designated *mut3* and *mut59* were obtained. The transposon insertion site was identified by transformation with pRSQ2-URA3 (3), digestion of the genomic DNA with EcoRI, ligation under dilute conditions, recovery in bacteria, and DNA sequencing of the transposon-insert junction.

Gene disruptions were carried out using a kanamycin resistance cassette generated from plasmid pRS400-KanMX4 as the template by a PCR-assisted method (55). PCR products were purified using a PCR purification kit (QIAGEN), and transformed into the diploid yeast strain GY763. Following transformation, yeast cells were grown in liquid YPD for 4 h and spread onto YPD plates containing G418

(200 μ g/ml; BRL) to select transformants that had the gene disrupted by the kanamycin cassette via homologous recombination.

Plasmids. A SacI-XhoI fragment from pBM3352 (22) containing *SET2* was subcloned into pRS416, generating pCYY23 (*CEN URA3 SET2*). The SacI-XhoI fragment containing *set2C82YD83Q* from pBM3354 (22) was subcloned into pRS416 to generate pCYY25 (*CEN URA3 set2C82YD83Q*).

Western blots. Yeast crude extracts were prepared by growing 30 ml of cells to a concentration of 2×10^7 to 3×10^7 cells per ml. Cells were harvested by centrifugation at 2,000 rpm for 5 min and resuspended in 500 μ l of breaking buffer (50 mM Tris [pH 7.5], 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, one tablet of protease cocktail [Roche]). Cells were disrupted by vortexing with glass beads, and extracts were clarified by centrifugation at $16,000 \times g$ for 10 min (59). Proteins were separated by 10 to 20% gradient polyacrylamide gel electrophoresis (PAGE) gel (Bio-Rad) and transferred to Immuno-Blot polyvinylidene difluoride membranes using a Bio-Rad Transblot SD transfer cell. Membranes were blocked in 3% bovine serum albumin (or 1% bovine serum albumin for mono-methyl K36 antibody) in PBS-Tween for 1 h and incubated overnight at room temperature with primary antibody followed by peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody.

The antibody complexes were detected using enhanced chemiluminescence reagents (Pierce) as specified by the manufacturer. Antibodies that recognize total histone H3 (ab1791), monomethylated histone H3 K36 (ab9048), and trimethylated histone H3 K36 (ab9050) were obtained from Abcam, and the antibody that recognizes dimethylated histone H3 K36 was obtained from Upstate (07-369). Western analysis for the antibody specificity test was performed as described above except that peptides were spotted onto polyvinylidene difluoride membranes and allowed to dry overnight. The membranes were incubated with primary antibody for 1 hour, followed by a 1-h incubation with secondary antibody. For *bur1*, *bur2*, and *ctk1* strains the amount of extract was increased 20 to 50% to adjust for the smaller amount of total histone H3 typically observed in those extracts.

Chromatin immunoprecipitations. Chromatin immunoprecipitations were performed essentially as described (27). Chromatin was sheared using an Ultrasonics Inc. sonicator at level three with 12 pulses of 20 seconds. Immunoprecipitations (IPs) were performed using 2.5 μ l antibody to monomethyl H3 K36, 1.0 μ l antibody to trimethyl H3 K36, and 1.0 μ l anti-H3 antibody. Inputs and IP samples were analyzed by standard PCRs to which 1 μ Ci of 32 P-labeled dATP had been added. Products were resolved on 1.8% agarose gels and quantified using a Storm 840 scanner and ImageQuant software (Molecular Dynamics). Results are normalized to inputs and expressed as ratios of methylated histone H3 K36 to total histone H3. The results shown are representative of several independent experiments that gave similar results.

Primers for *HMRA* and the *ACT1*, *PM1*, and *GAL10* open reading frames were as follows: *HMRA*, 5'CAGTTTCCCCGAAAGAACAA and 5'CCATCCG CCGATTATTT; *ACT1*, 5'CCAATTGCTCGAGAGATTTC and 5'CATGA TACCTTGGTGTCTTG; *PM1*, 5'AAGTCGTCCAGGTGATATTTGCA and 5'AACGAAAGTGTGTACCCGGTAGC; and *GAL10*, 5'GATCTTCCA TACAAAGTTACGG and 5'GGCCTCGACACCCC.

RESULTS

Genomic suppressors of *bur1* Δ . Deletion of *BUR1* was reported to cause lethality or extremely poor growth (13, 17, 42). In our strain background, *bur1* Δ strains grew extremely slowly, but revertants that grew significantly better arose readily, prohibiting propagation of pure *bur1* Δ strains. We anticipated that these viable *bur1* Δ revertants contained genomic suppressor mutations that would be informative for understanding the roles of *BUR1* in vivo. Although revertants of *bur1* Δ arose spontaneously, we utilized a transposon mutagenesis strategy to identify suppressors of *bur1* Δ and to facilitate rapid identification of the mutant genes. A *bur1* Δ strain, covered by a *BUR1*⁺ *URA3 CEN* plasmid to maintain viability, was transformed with an mTn-*lacZ-LEU2* integrating-plasmid library (3), selecting for transposon insertions that allowed loss of the *BUR1 URA3* plasmid.

The entire screening involved three steps. First, ~51,000 Leu⁺ transformants were screened for 5-FOA-resistant colo-

FIG. 1. Isolation of genomic suppressors of *bur1* Δ . (A) Strains with the indicated genotypes were streaked onto a YPD plate and grown for 4 days. The *bur1* Δ colonies are not visible at this time. Mutants 3 and 59 were subsequently found to contain transposon-linked suppressors, while the other strains contained uncharacterized spontaneous genomic suppressors. (B) PCR screening of revertant strains. Primers that are diagnostic for the *BUR1* locus were used in PCRs on revertant colonies. The *mut3* and *mut59* strains contained *bur1* Δ ::*TRP1* but not *BUR1*⁺. pGP105 is a control plasmid that contains *bur1* Δ ::*TRP1*. Lane M, AccI-digested λ DNA size markers.

nies, which could include the desired viable *bur1* Δ strains, plasmid-borne *ura3* mutations, or gene conversion of *bur1* Δ to *BUR1*⁺ (Fig. 1A). To distinguish between these possibilities, the 5-FOA-resistant colonies were screened by PCR amplification using primers that distinguish the presence of *bur1* Δ and *BUR1*⁺ (Fig. 1B). Finally, a genetic cross was utilized to determine whether the mutation responsible for any viable *bur1* Δ strains was linked to the *LEU2*⁺-marked transposon. Through this process we obtained two *bur1* Δ deletion strains in which there was no detectable source of *BUR1*⁺ and survival of which was strongly linked to the *LEU2*-marked transposon.

The insertion site was identified for both suppressors by rescuing the transposon and DNA sequencing of the transposon-insertion junction. Interestingly, both suppressors were due to insertions into *SET2*, which encodes a histone methylase that targets lysine 36 of histone H3 (22, 52). One insertion occurred after amino acid 136 in the SET domain that is required for methylase activity, while the other insertion was after amino acid 495 in the WW domain, suggesting that suppression was due to loss of *SET2* function. This conclusion was confirmed by showing that a *set2* deletion suppressed *bur1* Δ as efficiently as the original transposon insertion alleles (Fig. 2A) and that transformation with a *SET2*⁺ *CEN* plasmid reversed the suppression phenotype (Fig. 2B).

The identification of *set2* mutations as suppressing the severe growth defect of *bur1* Δ was important because, despite the widely inferred importance of histone methylation, *set2* deletion strains are healthy and display very few phenotypes; *set2* Δ has been reported to cause both 6-azauracil-sensitive and

FIG. 2. *set2* mutations suppress *bur1Δ*. (A) Strains with the indicated genotypes were streaked onto a YPD plate. The *set2Δ* strain suppresses the *bur1Δ* inviability as effectively as the original *mut3* and *mut59* transposon insertions, indicating that suppression is due to loss of *SET2* function. (B) A *bur1Δ set2Δ* strain was transformed with empty vector or a *SET2 CEN* plasmid. The *SET2 CEN* plasmid restored the poor growth indicative of *bur1Δ*, demonstrating that suppression is due to *set2Δ*.

6-azauracil-resistant phenotypes (18, 20, 25, 26, 47), and *set2Δ* increases transcription from a *gal4ΔUAS* allele (22). Although suppression of *gal4ΔUAS* by *set2Δ* appears analogous to our *Bur[−]* selection, which uses *suc2ΔUAS* as a reporter, *set2Δ* did not cause *Bur[−]* or *Spt[−]* phenotypes, which are typically associated with *bur1* and *bur2* mutations, and *bur1-8* did not reverse the *Gal[−]* phenotype of *gal4ΔUAS* (Fig. 3C and data not shown). These results suggest that although *SET2* and *BUR1* have independently been implicated in elongation, their roles are nonoverlapping, and instead are at least partially antagonistic.

Genetic analysis of the Bur1-Set2 relationship. A critical test for any suppressor is the specificity of the suppression phenotype (41), and the suppression of *bur1Δ* inviability by *set2Δ* was quite specific by several criteria. Two other histone methylases have been identified in yeast; Set1 methylates histone H3 on lysine 4 (2, 19, 32, 43) and Dot1 methylates H3 on K79 (34, 54). Deletion of either *SET1* or *DOT1* did not suppress *bur1Δ*, either singly or in combination, and *set1Δ* and *dot1Δ* also did not strengthen the suppression by *set2Δ* (Fig. 3A and data not shown). This demonstrates the specificity of the interaction and the lack of redundancy between the histone methylases, at least with respect to *BUR1*.

The specificity of suppression was tested in other ways. To

FIG. 3. Specificity of suppression. (A) Strains with the indicated genotypes were streaked onto a YPD plate. Note that *dot1Δ* and *set1Δ* have no effect on *bur1Δ*, while *set2Δ* suppresses the *bur1Δ* growth defect. (B) Strains with the indicated genotypes were replica plated to test for suppression of other *bur2Δ* phenotypes. *set2Δ* suppresses the formamide sensitivity (FA) and the cold-sensitive growth phenotype of *bur2Δ* but has no effect on the *Bur[−]* or *Spt[−]* phenotype, as judged by growth in YP plus sucrose or SC – histidine plates. (C) Suppression of *bur2Δ* growth defect by *set2Δ*. Fivefold dilutions of the strains listed were spotted onto YPD plates and photographed after 5 days.

test for gene specificity, double mutants were created with *set2Δ* and deletions of other genes that are involved in elongation. *set2Δ* reversed the formamide-sensitive and cold-sensitive phenotypes of *bur2Δ* mutants (Fig. 3B), had a modest effect on the growth of *bur2Δ* strains (Fig. 3C), and was unable to suppress the inviability caused by deletion of *SPT5*, *SPT6*, or *SPT16* (data not shown), each of which is phenotypically related to *BUR1* and has a role in elongation. Although we cannot rule out the possibility that *set2Δ* might suppress subtler mutations in these or other genes, suppression by *set2Δ* was specific for the Bur1-Bur2 complex and did not extend to any other elongation or transcription factors tested to date. Finally, *set2Δ* did not suppress all *bur1* or *bur2* mutations; *set2Δ* suppressed the temperature-sensitive phenotype of the *bur1-8* missense mutant (data not shown), the inviability of *bur1Δ* mutants, and the poor growth, formamide-sensitive, and *Cs[−]* phenotype of *bur2Δ* mutants, while other *bur1Δ* and *bur2Δ* phenotypes and healthier *bur1* or *bur2* alleles were not suppressed. Since *set2Δ* suppresses *bur1Δ* and *bur2Δ* null alleles, this example of allele specificity is not indicative of any physical interaction between the two proteins, but likely suggests that severe defects in the kinase complex are required to observe the relationship with *SET2*. Combined, these tests demonstrate the specificity of the relationship between the Bur1-Bur2 complex and *SET2*.

The identification of *set2Δ* as a suppressor of the *bur1Δ* growth defect functionally linked *BUR1* to histone methylation. But was suppression due to defective methylation of histone H3, or could suppression be due to some other unsuspected role of *SET2*? Two results clearly indicated that suppression was due to loss of *SET2*-dependent methylation of K36. First, a *set2 C82Y* missense mutation that causes defec-

FIG. 4. Suppression is due to loss of methylation of histone H3 on lysine 36. (A) A *bur1Δ set2Δ* strain was transformed with empty pRS416 vector or *CEN* plasmids containing *SET2*⁺ or a methylation-defective *set2* allele (*set2C82Y*). Growth of the transformed strain is indicated; transformation with *SET2*⁺ resulted in the nearly lethal *bur1Δ* phenotype, while *set2C82Y* was unable to complement the *set2Δ* suppression of *bur1Δ*. (B) Strains with the indicated genotypes were streaked onto a YPD plate. Note that the histone H3 K36R mutation suppresses *bur1Δ* as well as *set2Δ* does. WT, wild type.

tive methylation activity in vitro (22) suppressed *bur1Δ* as effectively as *set2Δ* (Fig. 4A), suggesting that loss of methylation activity was responsible for suppression. Second, if suppression of *bur1Δ* and *bur2Δ* was due to loss of methylation of histone H3 K36, then mutation of K36 should also suppress *bur1Δ*. As predicted, a histone H3 K36R mutation suppressed *bur1Δ* as effectively as *set2Δ*, strongly indicating that suppression of *bur1Δ* was caused by loss of K36 methylation (Fig. 4B).

Molecular analysis of the Bur1-Set2 relationship. To gain a better understanding of the relationship between *BUR1* and *SET2*, we investigated the effect of *bur1* mutations on the methylation status of histone H3 in vivo using antibodies that specifically recognize monomethylated, dimethylated, and trimethylated lysine 36 of histone H3. The specificity of the antibodies was first tested by assaying for whether the antibodies cross-reacted with unmodified or methylated histone H3 peptides, and as shown in Fig. 5A, each antibody was highly specific for the appropriate methylated peptide.

Using these antibodies in Western blots of crude cell extracts, we found that a combination of monomethylated, dimethylated, and trimethylated forms of lysine 36 was detected in wild-type strains, and as expected, all methylation was *SET2* dependent (Fig. 5B). In a temperature-sensitive *bur1-8* mutant strain, the levels of trimethylated K36 were significantly reduced, while monomethylated and dimethylated K36 persisted longer than in the *BUR1*⁺ strain, with the monomethylated K36 levels decreasing after prolonged incubation at the non-permissive temperature (Fig. 5B and C). This specific reduction of trimethylated K36 could be detected at the permissive temperature in the *bur1-8* strain and was exacerbated at 37°C, while methylation of K36 was unaffected in a wild-type strain at

FIG. 5. Trimethylation of H3 K36 is reduced in *bur1-8* and *bur2Δ* strains. (A) The methylated and unmethylated histone H3 K36 peptides listed at the top were spotted onto filters and subjected to Western blotting with the monomethyl-, dimethyl-, and trimethylation-specific antibodies listed below the panel. The antibodies used were for total histone H3 (ab1791), monomethylated K36 (ab9048), trimethylated K36 (ab9050), and dimethylated K36 (07-369) (B) Crude extracts were prepared from yeast strains with the genotypes shown at the top, and Western blots were performed using antibodies specific for total H3, monomethylated K36, dimethylated K36, and trimethylated K36. The strains on the right were shifted to 37°C for 3 h. The prominent lower band of the doublet in the panel labeled mono is a background band, since it does not respond to *set2Δ*. Note that all methylated forms are abolished in the *set2Δ* strain, while only the trimethylated form is significantly reduced in the *bur1-8* strain relative to total H3. WT, wild type. (C) Time course after shifting a *bur1-8* strain to the nonpermissive temperature reveals rapid loss of trimethylation.

either temperature. A similar reduction of trimethylated K36 was also observed in a *bur2Δ* strain (Fig. 5B). These results suggest that the Bur1-Bur2 kinase is directly or indirectly required for the transition from a dimethylated to trimethylated state of histone H3 at K36. This is analogous to the recently proposed requirement of Bur1 for the transition of K4 methylation from the dimethylated to the trimethylated state (23).

The Western blots shown in Fig. 5 assay the methylation status of total histone H3 in crude cell extracts. To analyze the K36 methylation state at specific genes, chromatin immunoprecipitation assays were performed using the monomethylated K36- and trimethylated K36-specific antibodies. Previous chromatin immunoprecipitation assays demonstrated that the K36 dimethylation pattern is unaffected by *bur2Δ* (57). At two constitutively highly expressed genes, *ACT1* and *PMAI*, the level of monomethylated K36 was essentially unchanged in *BUR2*⁺ versus *bur2Δ* strains, while the level of trimethylated K36 was significantly reduced, consistent with the results obtained in the crude extracts (Fig. 6A).

At two genes that are transcriptionally inactive under the growth conditions used, *HMR* and *GAL10*, the levels of trimethylated K36 were low and largely unaffected by the *bur2Δ* mutation. When *GAL10* was induced on galactose-containing medium, the level of trimethylated K36 increased significantly in a *BUR2*⁺ strain, but not in a *bur2Δ* mutant (Fig. 6B). The slight increase in trimethylated K36 observed in the *bur2Δ* strain is likely due to low levels of Bur2-independent Bur1

FIG. 6. Assaying methylation in *bur2Δ* mutants by chromatin immunoprecipitation. Wild-type and *bur2Δ* strains were grown to mid-log phase in YPD (panel A) or YP with glucose (Glu) or galactose (Gal) (panel B). Cross-linked extracts were immunoprecipitated with antibodies specific for total histone H3 or mono- or trimethylated K36. Chromatin immunoprecipitation analysis was done using primer sets specific for the transcriptionally silenced *HMR* locus and the open reading frames of the *GAL10*, *ACT1*, and *PMA1* genes. PCR products were quantified and are presented as the ratio of methylated H3 to total H3 for each sample. WT, wild type.

kinase activity or BUR-independent methylation by Set2. Combined, these results reveal that both at individual genes and in total cell extracts, the Bur1-Bur2 protein kinase is required for the transcription-associated increase in histone H3 K36 trimethylation, but not for K36 monomethylation or dimethylation.

To determine whether the reduction of trimethylated K36 was observed in other mutant backgrounds, we began by surveying a panel of *Spt*[−] mutants, which are phenotypically most closely related to *BUR1* mutants (Fig. 7A). A variety of patterns were observed; mutations in *SPT4* and *SPT5* (DSIF subunits) had no effect on K36 methylation, an *spt16* mutation selectively reduced trimethylation, although not to the extent observed in *bur1* or *bur2* strains, and *spt6* mutants showed allele-specific effects. An *spt6-140* strain displayed wild-type methylation in these crude extracts, while only the monomethylated form of K36 was observed in an *spt6-1004* strain. The reason for this allelic difference is currently unknown, but phenotypic differences between these two alleles have been observed previously; both strains display strong Ts[−], Bur[−], and Spt[−] phenotypes, but only the *spt6-1004* mutation increases initiation from cryptic promoters (15).

FIG. 7. Methylation changes in selected transcription factor mutant strains. (A) A panel of strains containing mutations in genes that cause phenotypes similar to that of *bur1* mutants were grown at 37°C for 3 h, and extracts were prepared and tested for K36 methylation levels by Western blotting with methylation-specific antibodies. The *spt16* strain displays reduced trimethylated K36, and the *spt6-1004* strain displays strong reduction of di- and trimethylated K36. WT, wild type. (B) A panel of strains containing mutations in genes responsible for histone modifications or that contain truncations in the CTD of Rpb1 were tested for methylation levels as in panel A. The level of total H3 in crude *ctk1Δ* extracts is typically lower than that of wild-type extracts. Nonetheless, the *ctk1Δ* strain has reduced levels of K36 of all three methylation states, while the three *rpb1* CTD truncations selectively reduce trimethylated K36 levels.

Strains containing mutations in genes related to *SET2* were examined next (Fig. 7B). Three patterns were observed again; a deletion of *CTK1*, which encodes a protein kinase previously reported to be required for K36 dimethylation (58), completely abolished all methylation of K36, similar to *set2Δ*, while deletion of *SET1*, *DOT1*, or *RAD6*, which are responsible for methylation of K4 and K79 of histone H3 and ubiquitylation of histone H2B, respectively, had no effect on K36 methylation. Finally, three truncations of the Rpb1 CTD (Fig. 7B) selectively reduced K36 trimethylation, as in *bur1* and *bur2* mutants, with the CTD truncations having a more severe effect than the *bur* mutations. Combined, these results suggest that the methylation pattern of K36 is affected by many factors in a complex manner, with the selective loss of K36 trimethylation being observed in *bur1*, *bur2*, *spt16*, and *rpb1* CTD truncation strains. More extensive methylation defects that differ from the pattern observed in *bur1* mutants occur in *spt6* and *ctk1* mutants.

Posttranslational modifications of histones have been proposed to affect nucleosome function by two broad mechanisms, either by directly affecting interactions with DNA and the other histones, or by affecting the recruitment of modification-specific histone-binding proteins (10). In particular, methylation marks on histone H3 on lysine 4, lysine 9, and lysine 27

FIG. 8. Suppression of *bur1Δ* by deletion of genes coding for chromodomain-containing proteins. (A) Strains with the indicated genotypes were streaked onto YPD plates and grown for 4 days. The *bur1Δ* strain is barely visible at this point. (B) Crude extracts were prepared from yeast strains with the genotypes shown at the top, and Western blots were performed using antibodies specific for total H3, mono-methylated K36, dimethylated K36, and trimethylated K36. The *set2Δ* and *bur1Δ set2Δ* strains have no detectable K36 methylation, while the *bur1Δ eaf3Δ* and *bur1Δ chd1Δ* strains are selectively reduced for trimethylated K36.

stimulate direct binding of Chd1, HP1, and polycomb (PC), respectively, via recognition of the methylated residues by the Chd1, HP1 and PC chromodomains (1, 5, 9, 21, 38). Based on these precedents, we expected that methylation of K36 recruits a chromodomain-containing protein.

Three chromodomain proteins, Chd1, Eaf3, and Esa1, have been identified in yeast (38), and all three have chromatin-mediated functions in transcription. As *CHD1* and *EAF3* are nonessential genes, we tested whether deletion of either of these genes could also suppress *bur1Δ*, and indeed, *chd1Δ* and *eaf3Δ* individually suppressed the *bur1Δ* growth defect (Fig. 8A). Unlike *set2Δ*-mediated suppression, which reduced all K36 methylation, the methylation pattern in *bur1Δ eaf3Δ* and *bur1Δ chd1Δ* strains was unchanged compared to that of *bur1Δ* mutants, with normal levels of mono- and dimethylated K36 but greatly reduced trimethylation (Fig. 8B). We conclude that *eaf3Δ* and *chd1Δ* suppress *bur1Δ* differently than *set2Δ* does; while *set2Δ* results in loss of methylated K36 marks, *eaf3Δ* and *chd1Δ* abolish downstream effects of K36 mono- or dimethylation. In agreement with these results, a recent report (16) demonstrated that *bur1Δ* is suppressed by *eaf3Δ* and *chd1Δ*.

DISCUSSION

The major conclusion from this study is that a functional connection exists between Bur1 and histone methylation in vivo, based on the following evidence: deletion of *SET2*, which encodes the histone H3 K36 methylase, reverses the poor growth of *bur1Δ* cells; the severe growth defect of *bur1Δ* cells

is due to Set2-dependent methylation of histone H3 on K36; and trimethylated K36 is greatly reduced in *bur1Δ* and *bur2Δ* mutant strains. Although previous studies independently linked the Bur1-Bur2 Cdk with chromatin and transcription elongation, and also suggested a role for Set2-mediated methylation of K36 during elongation, the importance of our results is that they bridge these two areas of research and suggest a closer regulatory relationship between Bur1 and Set2 than had been appreciated.

The ability to isolate suppressor mutations that restore growth to *bur1Δ* strains indicates that the Bur1-Bur2 Cdk complex is not intrinsically required for viability, but instead is part of a pathway that is required to overcome a strong growth-inhibitory effect that occurs through Set2-dependent methylation of histone H3. Two questions immediately emerge from this simple genetic result. First, what genes and molecular events are responsible for the near-lethal phenotype in *bur1Δ* strains, and second, what is the specific role of the Bur1-Bur2 Cdk complex in overcoming the inhibitory effect? Although we cannot completely answer these questions, the results presented here, combined with recent results from other groups, provide insight into these issues.

With regard to the first question, it is clear that the growth defect is caused by *SET2*-dependent methylation of histone H3 on K36, as a *SET2* deletion or a histone H3 K36R mutation suppresses *bur1Δ* equivalently. Since we observed only mono- and dimethylated K36 in *bur1Δ* and *bur2Δ* strains, we further infer that the growth defect is mediated through either mono- or dimethylated K36. Our interpretation is that *set2Δ* suppresses *bur1Δ* because in that mutant background loss of methylation is less detrimental to the cell than partial or incomplete methylation.

Histone modifications do not always act in isolation, but instead are believed to recruit downstream effectors that recognize specifically modified histones. Our results indicate that K36 mono or dimethylation is required but is not sufficient for inhibiting the growth of *bur1Δ* strains, as deletion of either *EAF3* or *CHD1*, both of which encode chromodomain-containing factors, also suppresses *bur1Δ*, indicating that they are nonredundant components of the inhibitory pathway.

Importantly, the mechanism of *eaf3Δ*- and *chd1Δ*-mediated suppression of *bur1Δ* differs from that of *set2Δ*, as *bur1Δ eaf3Δ* and *bur1Δ chd1Δ* strains have normal levels of mono- and dimethylated K36, but no detectable trimethylated K36. Moreover, deletion of *EAF3* or *CHD1* does not enhance suppression of the *bur1Δ* growth defect by *set2Δ* (data not shown), suggesting that Eaf3, Chd1, and methylated K36 are part of the same pathway. We conclude that Eaf3 and Chd1 are downstream effectors of the mono- or dimethylated K36 marks. Consistent with these results, additional mechanistic insight into the role of *EAF3* emerged from recent studies demonstrating that Eaf3 binds directly to histone H3 peptides through its chromodomain, and that binding of Eaf3 is modestly stimulated by dimethylation of K36 (6, 14). A stronger effect of K36 methylation on recruitment of Eaf3 was observed by using nucleosomes and the Eaf3-containing Rpd3S complex, suggesting that other interactions might be involved (16). SET2-dependent methylation of K36 thereby results in recruitment of the Rpd3S histone deacetylase complex to the coding region of transcribed genes, generating a localized deacetylated tem-

plate that is proposed to contribute to restoring transcriptionally active genes to an inactive state (6).

The mechanism of suppression by *chd1Δ*, however, is less clear. Chd1 has been reported to bind to dimethylated K4 of histone H3 via the Chd1 CD2 chromodomain (38), although this conclusion has been questioned by other authors (11, 51). This proposed K4 methyl-binding activity of Chd1 does not easily account for the ability of *chd1Δ* to suppress *bur1Δ*, however, as deletion of *SET1*, which is responsible for methylating K4, did not suppress *bur1Δ* alone or in combination with *set2Δ* (Fig. 3A), and a histone H3 K4R mutation does not suppress *bur1Δ* (16). The detailed role of *CHD1* in suppression of *bur1Δ* thus still remains uncertain at the molecular level, but our results suggest that it functions at least partially through methylated K36. Since Chd1 contains two chromodomains, it remains possible that Chd1 has dual specificity for binding methylated K4 and K36.

The second major question arising from this study is how the Bur1-Bur2 kinase stimulates K36 trimethylation. One model is that *BUR1* could affect trimethylation by directly phosphorylating and regulating Set2. A second model is that the effects of the *bur1* mutations on trimethylation of K36 could simply be an indirect result of altered transcription in these strains. A third model is that Bur1 could indirectly regulate Set2 activity or a functional association of Set2 with polymerase II or other transcription factors.

The first model is unlikely. Although we found that the purified Bur1-Bur2 complex could directly phosphorylate recombinant Set2 in vitro, mutation of the phosphorylation sites in Set2 had no effect on the growth of cells in *BUR1⁺* and *bur1Δ* strains or on the K36 methylation state (data not shown). For the second model, the finding that K36 trimethylation is only affected in specific transcription factor mutant strains, not all of them, indicates that the changes in K36 trimethylation are not merely a result of altered transcription, but instead suggests a more specific connection between *BUR1* and K36 trimethylation. Based on these results and the finding that *rpb1* CTD truncations and an *spt16* mutation affect the di- to trimethyl transition, we currently favor the third model, in which phosphorylation of an intermediate factor promotes Set2-dependent trimethylation.

Three in vitro substrates of the Bur1-Bur2 kinase have been identified to date: Spt5 (36), the polymerase II CTD (31), and Rad6 (57). These substrates might not be responsible for promoting K36 trimethylation, since *set2Δ* does not suppress *spt5Δ* and *spt5-194* does not affect the K36 methylation profile, CTD phosphorylation is reported to be unaffected in *bur1* mutants (17), and *rad6Δ* does not affect the K36 methylation profile. Based on their phenotypes, possible targets include Spt16, Eaf3, and Chd1, while a hypothetical K36-specific demethylase and histone H3 itself remain reasonable candidates.

During the process of testing whether other mutations cause effects similar to those of *bur1* and *bur2* mutations on the histone H3K36 methylation state, it became apparent that effects on the K36 methylation state are complex. Although several proteins have already been described as being required for methylation of K36, including Ctk1 and a phosphorylated polymerase II CTD (20, 25, 26, 58), those studies only examined effects on dimethyl K36. We expanded on these results and found that deletion of *SET2* and *CTK1* uniformly reduced

all forms of methylated K36; mutations in *SPT4*, *SPT5*, *SET1*, *DOT1*, and *RAD6* had no detectable effect on K36 methylation; while *bur1*, *bur2*, and *spt16* mutants and *rpb1* CTD truncations primarily reduced K36 trimethylation. In addition, *spt6* mutations caused allele-specific effects on the K36 methylation state, with the *spt6-140* strain displaying nearly wild-type methylation, and the *spt6-1004* strain only containing detectable monomethylated K36. It will be interesting and informative to determine whether mutations that affect other relatively general transcription factors show widespread effects on K36 methylation, but such an issue will be best addressed using large-scale systematic screening methods, such as the global proteomic system that was utilized to identify factors involved in H2B ubiquitylation and K4 methylation (48).

The functional relevance of K36 methylation for transcriptional regulation remains an open issue, largely resting upon the association of di- and trimethylated K36 with the 3' end of open reading frames in chromatin IP assays (20, 37, 52). The importance of this K36 methylation pattern is not immediately obvious, however, since *set2Δ* and histone H3 K36R mutants are relatively healthy in an otherwise wild-type background. Our results and the recent report from Keogh et al. (16) point to a functional importance of K36 methylation. More specifically, the identification of factors that are required for the transition from di- to trimethylated K36 implies regulatory events and a functional significance for this step.

Precedents for functional distinctions based on the number of methyl groups have been described for Set1-mediated methylation at K4 of histone H3, in which trimethylated K4 is specifically associated with actively transcribed genes in yeast (45), and the Lsd1 demethylase is active on dimethylated but not on trimethylated K4 (50), but functional distinctions based on the number of methyl groups at K36 in *S. cerevisiae* are only beginning to emerge (30). The results presented here further expand our view of the role and regulation of histone methylation.

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