Phosphorylation of RNA polymerase II CTD regulates H3 methylation in yeast

Tiaojiang Xiao, Hana Hall, Kelby O. Kizer, Yoichiro Shibata, Mark C. Hall, Christoph H. Borchers, and Brian D. Strahl[1]

Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599, USA

Histone methylation is now realized to be a pivotal regulator of gene transcription. Although recent studies have shed light on a *trans*-histone regulatory pathway that controls H3 Lys 4 and H3 Lys 79 methylation in *Saccharomyces cerevisiae*, the regulatory pathway that affects Set2-mediated H3 Lys 36 methylation is unknown. To determine the functions of Set2, and identify factors that regulate its site of methylation, we genomically tagged Set2 and identified its associated proteins. Here, we show that Set2 is associated with Rbp1 and Rbp2, the two largest subunits of RNA polymerase II (RNA pol II). Moreover, we find that this association is specific for the interaction of Set2 with the hyperphosphorylated form of RNA pol II. We further show that deletion of the RNA pol II C-terminal domain (CTD) kinase Ctk1, or partial deletion of the CTD, results in a selective abolishment of H3 Lys 36 methylation, implying a pathway of Set2 recruitment to chromatin and a role for H3 Lys 36 methylation in transcription elongation. In support, chromatin immunoprecipitation assays demonstrate the presence of Set2 methylation in the coding regions, as well as promoters, of genes regulated by Ctk1 or Set2. These data document a new link between histone methylation and the transcription apparatus and uncover a regulatory pathway that is selective for H3 Lys 36 methylation.

DNA transcription is a highly coordinated and multistep process utilizing a large number of basal and transactivating factors. Within eukaryotic cells, however, DNA is packaged into chromatin, thereby creating an environment that is not readily permissive to these and other protein complexes that require access to the DNA. Whereas considerable progress has been made on the accessibility of DNA during initiation of transcription, much less is known about the mechanisms that regulate chromatin accessibility during transcription elongation (Orphanides and Reinberg 2000, 2002; Hartzog et al. 2002).

Posttranslational modifications of histone N termini are recognized to play a fundamental role in the structure and function of chromatin. The histone tail domains, which extend away from the nucleosome core particle, are subjected to a variety of modifications including acetylation, phosphorylation, and methylation (van Holde 1989; Grant 2001; Berger 2002). From recent work, it is becoming increasingly clear that these modifications form a histone code that regulates chromatin function through their ability to recruit specific interact-ing proteins that recognize a single or combinatorial set of modifications (Strahl and Allis 2000; Turner 2000; Jenuwein and Allis 2001). Examples of such regulation include the interaction of bromodomains with acety-lated histones (Filetici et al. 2001), and more recently, chromodomains with methylated lysines (Lachner and Jenuwein 2002). It is likely that the selective recruitment of downstream factors to chromatin will be a major mechanism of chromatin function that will extend to other histone modifications.

Histone methylation occurs on lysine and arginine residues (Zhang and Reinberg 2001). Regarding lysine methylation, studies show that a number of lysines (4, 9, 27, 36, and 79 of H3 and 20 of H4) are the major identified sites of methylation (van Holde 1989; Strahl et al. 1999; Khan and Hampsey 2002), although species-specific differences exist. Of importance, is the fact that each modified lysine can be mono-, di-, or trimethylated, and recent insights are revealing that these modification states evoke specialized biological outcomes (von Holt et al. 1989; Santos-Rosa et al. 2002). Whereas the existence of histone methylation was known for over three decades, only within the last several years has its functional significance begun to be revealed. This is due, in part, to the identification of the enzymes responsible for methylating histones and the generation of immunological tools to study the selective sites that become methylated (Zhang and Reinberg 2001; Kouzarides 2002;

[1]**Corresponding author.**
E-MAIL Brian_Strahl@med.unc.edu; FAX (919) 966-2852.

Lachner and Jenuwein 2002). Collectively, these studies reveal that histone methylation, and the enzymes that mediate it, play a fundamental role in the organization of chromatin and in the activation and repression of genes.

We and others recently showed the existence of a *trans*-histone regulatory pathway involving the ubiquitination of H2B in the regulation of H3 Lys 4 and Lys 79 methylation, but not H3 Lys 36 methylation in *Saccharomyces cerevisiae* (Briggs et al. 2002; Dover et al. 2002; Ng et al. 2002; Sun and Allis 2002). In this work, we reveal a novel pathway involving the phosphorylation of the C-terminal domain (CTD) in RNA polymerase II (RNA pol II) leading to the selective regulation of H3 Lys 36 methylation, but not of H3 Lys 4 or Lys 79 methylation in yeast. This pathway was uncovered initially through the biochemical purification of the H3 Lys 36 methyltransferase Set2, which in agreement with others (Li et al. 2002, 2003; N.J. Krogan, M. Kim, A. Tong, A. Golshani, G. Cagney, V. Canadien, D. Richards, B. Beattie, A. Emili, C. Boone, A. Shilatifard, S. Buratowski, and J. Greenblatt, in prep.; H.P. Phatnani and A.L. Greenleaf, in prep.), reveals this protein to be a phospho-CTD-binding protein of RNA pol II. Surprisingly, we show that deletion of the CTD kinase Ctk1, or partial deletion of the CTD domain itself, results in a total abolishment of H3 Lys 36 methylation, but not of Lys 4 or Lys 79 methylation. These data document an unexpected and selective pathway for the regulation of H3 Lys 36 methylation that links this modification directly with the transcription elongation process. Consistent with this hypothesis, we have determined that Set2 methylates at the coding and promoter regions of genes.

Results

Set2 is associated with hyperphosphorylated RNA pol II

Recent studies have identified Set2 as a nucleosomal-selective histone methyltransferase (HMT) that methylates the H3 tail domain at Lys 36 via its conserved SET domain (Strahl et al. 2002). Given that the functions of this enzyme are not well defined, we genomically tagged Set2 at the C terminus with a triple-Flag affinity sequence and used this tag to purify Set2-associated proteins. Wild-type or Set2-3Flag whole-cell extracts were prepared from asynchronously growing *S. cerevisiae* cells and incubated with anti-Flag beads. Following elution of Set2-3Flag using 3×Flag peptide, SDS-PAGE analysis revealed several high molecular weight proteins (220 and 140 kD) that were not observed in the wild-type control purification (Fig. 1A). These bands were gel excised, digested with trypsin, and the resulting peptides examined by mass spectrometry. Results of this analysis revealed these proteins to be Rpb1 and Rpb2 (Fig. 1A). Independently, we confirmed these interactions with Set2 in a different strain background that used the tandem affinity purification (TAP) tagging method (data not shown).

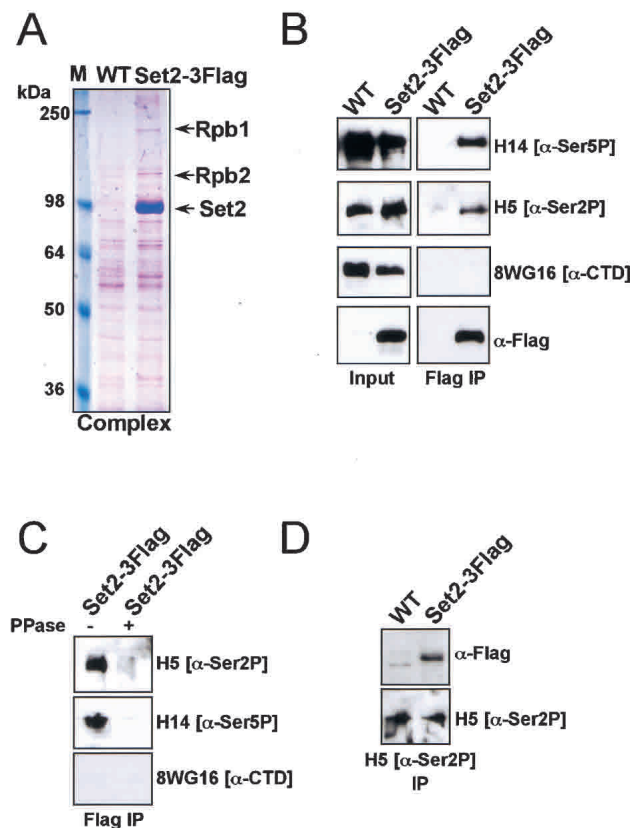Based on several reports that yeast WW domains (as is

Figure 1. Set2 interacts with RNA pol II. (*A*) Purification of Set2-3Flag. Wild-type (WT) or genomically tagged Set2 whole-cell extracts (WCE) were incubated with anti-Flag resin, and the resulting bound proteins eluted with 3×Flag peptide. Eluted proteins were resolved by 8% SDS-PAGE and examined by Coomassie staining. Arrows indicate the protein identity of bands that were examined by mass spectrometry; sizes of the molecular weight markers are shown. (*B*) Interaction of Set2 with hyperphosphorylated RNA pol II. WCE from wild-type or Set2-3Flag cells were immunoprecipitated with anti-Flag beads followed by immunoblotting with antibodies directed against unmodified CTD (8WG16 [α-CTD]), Ser 2-phosphorylated CTD (H5 [α-Ser2P]), Ser 5 phosphorylated CTD (H14 [α-Ser5P]), or α-Flag. These WCEs (Inputs) were also examined by immunoblot analysis with the antibodies described above to monitor the presence of these proteins. (*C*) Interaction of Set2 with RNA pol II is phosphorylation dependant. Flag immunoprecipitations (IPs) from wild-type or Set2-3Flag WCEs were separated and treated with either buffer only or buffer plus λ phosphatase (PPase) followed by immunoblotting with the H5 [Ser2P], H14 [Ser5P], and 8WG16 [α-CTD] antibodies. (*D*) Set2 coimmunoprecipitates with hyperphosphorylated RNA pol II. Wild-type or Set2-3Flag WCEs were first immunoprecipitated with the H5 [Ser2P] or H14 [Ser5P] antibodies, followed by immunoblot analysis with α-Flag. Results with both antibodies were identical and only the H5 [Ser2P] results are shown. We note that with our immunoprecipitations using CTD antibodies, we were able to efficiently detect Ser 2 phosphorylation from H14 [Ser5P] immunoprecipitations (and vise versa), indicating the heterogeneity of phosphorylation that exists on RNA pol II in vivo (data not shown). Thus, whereas Set2 may be interacting preferentially with Ser 2-phosphorylated CTD (see Fig. 5 and text), it is likely that both phosphorylation states would be detected in these assays.

found in Set2) bind to the phosphorylated CTD of RNA pol II (Morris et al. 1999; Morris and Greenleaf 2000), we next asked whether Set2 would be associated with the hyperphosphorylated form of RNA pol II. Using antibodies that distinguish between the unmodified CTD or CTD-phosphorylated at Ser 2 or Ser 5, we found that RNA pol II that associates with immunoprecipitated Set2-3Flag is phosphorylated at both Ser 2 and Ser 5 (Fig. 1B). Importantly, no unphosphorylated RNA pol II could be detected from these same immunoprecipitations, suggesting that this association is phosphorylation-dependent and likely a result of the fact that Set2 binds directly to the phosphorylated CTD (Fig. 1B). In support, we immunoprecipitated Set2-3Flag and treated the resulting immunoprecipitations with λ phosphatase (PPase). As analyzed by immunoblot analysis using the Ser 2 and Ser 5 phospho-specific antibodies, addition of λ phosphatase with immunoprecipitated Set2-3Flag resulted in a complete abolishment of the interaction of Set2 with RNA pol II (Fig. 1C). Importantly, unmodified CTD was not detected in the PPase-treated immunoprecipitations, indicating that the loss of CTD phosphorylation results in a dissociation between Set2 and RNA pol II (Fig. 1C). Finally, we confirmed the interaction of Set2 with RNA pol II in a reverse fashion by first immunoprecipitating RNA pol II with the Ser 2 or Ser 5 phospho-specific CTD antibodies followed by immunoblotting with anti-Flag (Fig. 1D; data not shown). These results demonstrate that Set2 interacts with the hyperphosphorylated form of RNA pol II, thereby revealing an intriguing and unexpected link between a histone methyltransferase and the transcription machinery.

The C terminus of Set2 interacts with the hyperphosphorylated form of RNA pol II

We next examined the regions in Set2 that are required for its interaction with RNA pol II. Full-length Set2-Flag, Set2-Flag containing a deletion of the WW domain, or Set2-Flag constructs containing N- or C-terminal deletions (Fig. 2A) were expressed in wild-type or yeast cells deleted in Set2 (set2Δ) and whole-cell extracts were prepared for coimmunoprecipitation studies. As expected, immunoprecipitation of wild-type full-length Set2-Flag followed by immunoblotting with the Ser 2 or Ser 5 phospho-specific CTD antibodies resulted in the detection of RNA pol II (Fig. 2B). Analysis of a construct containing only the N terminus of Set2 (residues 1–261) failed to show this interaction, indicating that the C terminus of Set2 functions in the binding of this protein to RNA pol II (Fig. 2B). Unexpectedly, a precise deletion of the WW domain in full-length Set2 did not abolish this interaction, indicating that other C-terminal regions in Set2 bind to RNA pol II (Fig. 2B). In agreement, a C-terminal region of Set2 (amino acids 533–733) that lacked the WW domain but still contained the coiled-coil motif was found to interact efficiently with RNA pol II (Fig. 2B). Given independent results from H.P. Phatnani and A.L. Greenleaf (in prep.) show that the WW domain of Set2, in isolation, has the capacity to interact
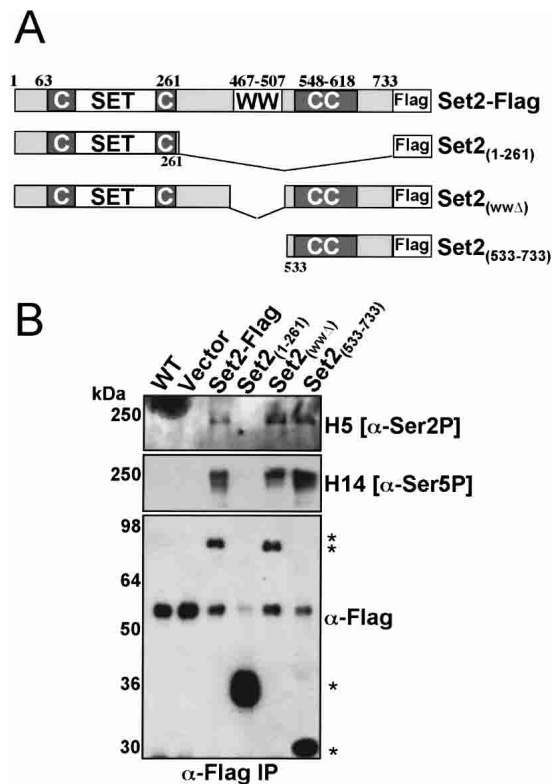

Figure 2. The C terminus of Set2 interacts with RNA pol II. (A) Schematic representation of the Set2-Flag constructs used to probe for RNA pol II interaction. The SET domain along with its flanking Cys-rich domains (C), WW domain (WW), and coiled-coil motif (CC) are shown. (B) set2Δ cells were transformed with either vector only or the indicated Set2-Flag constructs and WCEs were prepared. These extracts were immunoprecipitated with the α-Flag antibody followed by immunoblot analysis with the H5 [α-Ser2P], H14 [α-Ser5P], or α-Flag antibodies. Sizes of the molecular weight markers are shown, and asterisks indicate the location of expected Set2-Flag products. All input extracts showed equivalent levels of hyperphosphorylated RNA pol II (data not shown).

with Ctk1-phosphorylated CTD, it may be that the WW domain is sufficient, but not required, for interaction of Set2 with RNA pol II. More detailed studies will be necessary to define the precise regions of Set2 that contribute to this interaction.

Set2 methylates at the coding and promoter regions of genes

Given the finding that Set2 is binding to phosphorylated RNA pol II, and thus, is likely traveling with elongating complexes, we next asked whether Set2 methylation would be associated in the promoters and/or coding regions of genes. Using the H3 di-methyllysine 36 antibody, we performed chromatin immunoprecipitation (ChIP) assays in wild-type and set2Δ cells. Although the target genes of Set2 are not fully known, we analyzed a subset of genes that were either known targets of Set2

(e.g., *GAL4*) or those that were affected by *CTK1* (Patturajan et al. 1999; Cho et al. 2001; J. Landry, T. Hesman, J. Min, R.-M. Xu, M. Johnston, and R. Sternglanz, in prep.). Using primer sets that corresponded to either the promoter or coding regions of the genes indicated, we found that Set2 methylation was present in both regions (Fig. 3). To verify that the H3 Lys 36 methylation detected in the promoter regions is not a property of our DNA fragments containing coding-region sequences, we used RNA pol II CTD modification-specific antibodies in our ChIP assays that distinguish between the promoter and coding regions of genes (Komarnitsky et al. 2000). As expected, CTD phosphorylation at Ser 5 was detected only in the promoter regions of the genes we examined, whereas Ctk1-phosphorylated CTD (Ctk1 mediates Ser 2 CTD phosphorylation, see text) was found only in the

coding regions (Fig. 3). In addition, the unmodified CTD antibody showed the presence of RNA pol II in both the promoter and coding regions of the genes examined similar to what has been reported previously (data not shown; for review, see Komarnitsky et al. 2000). These results demonstrate the presence of H3 Lys 36 methylation in both the promoter and coding region of genes, thereby suggesting that Set2 is assembled with RNA pol II early in the transcription initiation process.

In addition to the genes shown, we also found the promoter and coding regions of the *ADH1*, *ENO1*, *SSA3*, and *CTT1* genes methylated by Set2 (data not shown). However, not all genes or distinct regions of chromatin we examined, including the *HIS3* gene and the rDNA and telomeres, were found to be methylated at H3 Lys 36 (data not shown), suggesting Set2 methylation is gene
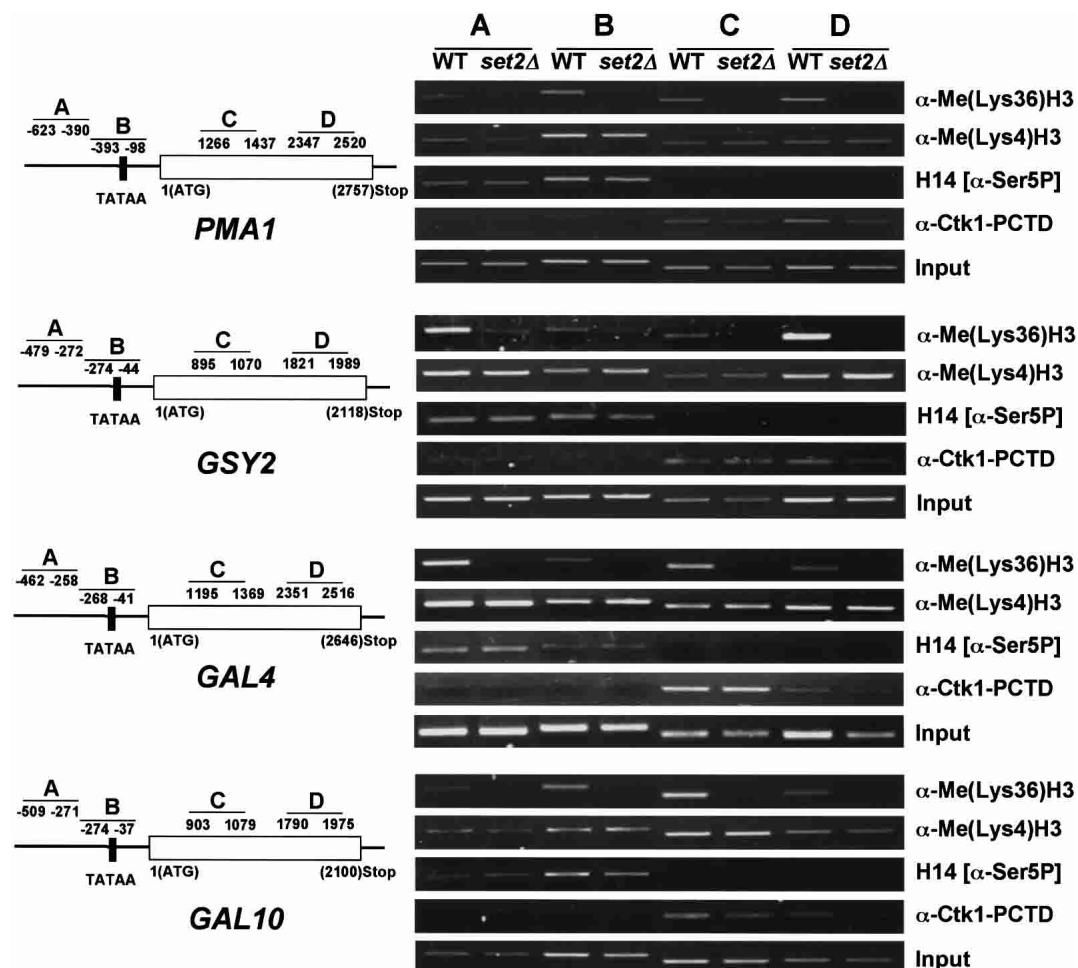

Figure 3. Set2 methylates at the coding regions, as well as promoters, of genes. Chromatin immunoprecipitation assays were used to examine the H3 Lys 36 methylation status on several genes in vivo. Whole-cell extracts prepared from formaldehyde-fixed wild-type or *set2Δ* cells were sonicated to shear their chromatin and then immunoprecipitated with either the H3 Lys 36 methylation-specific antibody [α-Me(Lys 36)H3], the H3 Lys 4 methylation-specific antibody [α-Me(Lys 4)H3], the CTD Ser 5 phosphorylation-specific antibody (H14 [α-Ser5P]), or the Ctk1-phosphorylated CTD antibody (α-Ctk1-PCTD). DNA from the enriched precipitates was isolated and used in PCR reactions with promoter or coding-specific primer pairs for the genes indicated. Primer pairs (labeled as A–D) include their sequence location relative to the translation initiation site. Although we show several genes positive for the presence of H3 Lys 36 methylation, other genes or distinct regions of chromatin examined (*HIS3*, rDNA, and telomeres) showed no enrichment for H3 Lys 36 methylation above background levels observed in *set2Δ* (data not shown).

selective. As a comparison with H3 Lys 36 methylation, we also analyzed these same genes for H3 Lys 4 methylation in wild-type and *set2Δ* using the anti-H3 di-methyllysine 4-specific antibody. Results showed the presence of this histone modification in all genes tested, and these levels did not change significantly in *set2Δ* cells (Fig. 3).

The CTD is essential for H3 Lys 36 methylation

To determine whether Set2's association with RNA pol II is physiologically relevant, we analyzed H3 Lys 36 methylation in a series of yeast strains in which the CTD was increasingly deleted (Nonet et al. 1987). Strikingly, results showed that a progressive deletion of the CTD in RNA pol II resulted in a progressive loss of H3 Lys 36 methylation (Fig. 4A). Importantly, other sites of histone methylation and Set2 mRNA levels as detected by reverse transcriptase PCR (RT–PCR) were unaffected by these CTD deletions (Fig. 4A,B), indicating the strong likelihood that Set2 is targeted to chromatin in vivo through recruitment by the CTD of RNA pol II. These

Figure 4. The CTD is required for H3 Lys 36 methylation. (*A*) Yeast nuclear extracts prepared from nondeleted CTD (Z26 and Z27), or the indicated CTD deletion strains were probed with antibodies against either methylated lysine residues at H3 Lys 36, Lys 4, and Lys 79 or acetylated H3. C3, C23, and V26 represent CTDs of ~10, 12, and 18 repeats, respectively (for review, see Nonet et al. 1987). Asterisk indicates partial N-terminal H3 breakdown products that are typically observed. (*B*) Expression levels of *SET2* or *ACT1* (used as a control) were not changed in the CTD tail-deletion strains. Shown are reverse transcription PCR reactions with (+) or without (–) reverse transcriptase (RTase).

results strongly suggest an important function for Set2 and Lys 36 methylation in transcription elongation.

The CTD kinase Ctk1 regulates H3 Lys 36 methylation

Given that the CTD is essential for H3 Lys 36 methylation, we next examined available deletion strains of known CTD kinases and proteins involved in transcription elongation to determine whether any of these factors might be responsible for targeting Set2 to RNA pol II and thereby regulate global H3 Lys 36 methylation. Surprisingly, we found that deletion of the CTD kinase Ctk1 resulted in a complete abolishment of global Lys 36 methylation (Fig. 5A). In contrast, deletion of the CTD kinase Srb10, or other proteins related to transcription elongation, did not result in any loss of H3 Lys 36 methylation (Fig. 5A). As with the CTD tail deletions, other sites of histone methylation and Set2 mRNA levels were unaffected by deletion of *CTK1* (Fig. 5A,C). As well, the loss of H3 Lys 36 methylation in the *CTK1* deletion strain could be restored with ectopically expressed Ctk1 (Fig. 5B). Given that Ctk1 is thought to be the major, if not exclusive Ser 2 CTD kinase (Kobor and Greenblatt 2002; Prelich 2002), our results suggest that Ser 2 phosphorylation, but not Ser 5 phosphorylation, is essential for recruitment of Set2 to RNA pol II.

Ctk1 activity is dependent on two other tightly associated factors that make up the Ctk1 complex, Ctk2 and Ctk3 (Sterner et al. 1995; Hautbergue and Goguel 2001). In examination of these other members, we found that deletion of *CTK2* and *CTK3* also results in the specific abolishment of H3 Lys 36 methylation (Fig. 5D), confirming the importance of these other components for Ctk1 activity. More recent evidence suggests that the Ctk1 complex also contains Sse2 (Gavin et al. 2002). Using H3 Lys 36 methylation as an indicator of Ctk1 function, our results suggest that this member is not essential for Ctk1 activity, as no change was observed with H3 Lys 36 methylation (Fig. 5D). Consistent with this conclusion is the fact that deletion of *SSE2* does not result in a significant slow growth phenotype as is observed from *CTK1* deletion strains (data not shown).

To determine whether the Ctk1 pathway of H3 Lys 36 methylation functions uni- or bi-directionally, we examined whether deletion of *SET2* might alter the phosphorylation status of RNA pol II. Results showed that whereas deletion of *CTK1* resulted in the complete abolishment of CTD Ser 2 phosphorylation, deletion of *SET2* did not affect the levels of Ser 2 or Ser 5 phosphorylation, indicating that this pathway is unidirectional (data not shown). Collectively, our data document an unexpected and novel pathway leading to the site-selective methylation of H3 Lys 36. Given that *CTK1* does not affect H3 Lys 4 or Lys 79 methylation, but that these sites of histone methylation are regulated in a distinct pathway by Rad6/H2B ubiquitination, we propose that site-selective histone methylation patterns are generally governed by distinct upstream events.
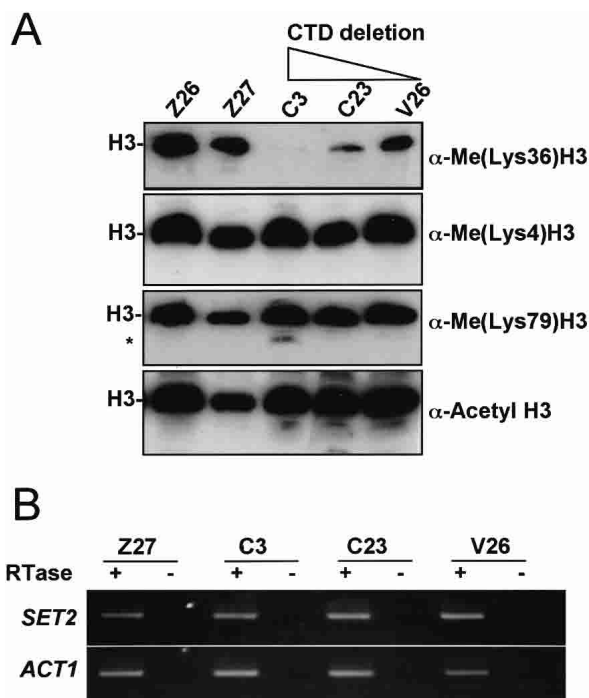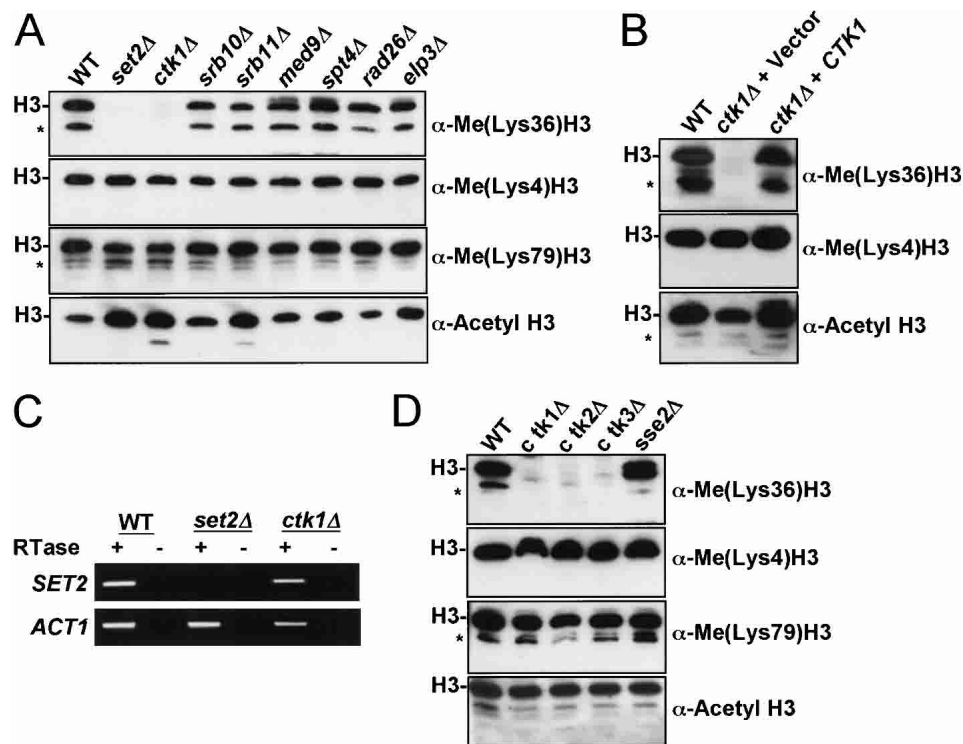
Figure 5. The CTD kinase Ctk1 is essential for Set2 methylation. (*A*) Yeast nuclear extracts prepared from wild-type or the indicated deletion strains were probed with antibodies against either methylated lysine residues at H3 Lys 36, Lys 4, and Lys 79 or acetylated H3. (*B*) Nuclear extracts isolated from wild-type or *ctk1*Δ strains containing either the vector control plasmid (*ctk1*Δ + Vector) or wild-type *CTK1* expression plasmid (*ctk1*Δ + *CTK1*) were probed with antibodies against either methylated lysine residues at H3 Lys 36 and Lys 4 or acetylated H3. (*C*) Expression levels of *SET2* or *ACT1* (used as a control) were not changed in *ctk1*Δ. Shown are RT–PCR reactions with (+) or without (–) reverse transcriptase (RTase). (*D*) The Ctk1 complex is essential for H3 Lys 36 methylation. Nuclear extracts isolated from wild-type or *ctk1*Δ, *ctk2*Δ, *ctk3*Δ, or *sse2*Δ strains were probed with antibodies against either methylated lysine residues at H3 Lys 36, Lys 4, and Lys 79 or acetylated H3. Asterisks indicate partial N-terminal H3 breakdown products that are typically observed. Sse2 was identified recently as a new component of the Ctk1 complex (Gavin et al. 2002).

Discussion

To further address the role of Set2 in *S. cerevisiae*, we have used an affinity-purification approach to identify Set2-associated proteins. In this study, we show that Set2 copurifies with the two largest subunits of RNA pol II. Furthermore, this interaction is specifically dependent on the binding of the C terminus of Set2 with the hyperphosphorylated state of RNA pol II CTD. Importantly, we find that H3 Lys 36 methylation, but not H3 Lys 4 or Lys 79 methylation, is dependent on the CTD domain as well as the specific CTD kinase Ctk1. Taken together, these results indicate a function for Set2, albeit undefined, in the transcription elongation process. In support, H3 Lys 36 methylation is found in the coding regions and promoters of genes regulated by Ctk1 or Set2.

A novel pathway for H3 Lys 36 methylation

Recent efforts have resulted in the characterization of a number of new histone methyltransferases and the upstream molecular pathways that are required for their methylation events (Zhang and Reinberg 2001; Henry

and Berger 2002; Kouzarides 2002; Lachner and Jenuwein 2002). Whereas Set1-mediated Lys 4 and Dot1-mediated Lys 79 methylation are regulated by Rad6 ubiquitination of H2B in a *trans*-histone regulatory pathway that controls gene silencing in yeast, H3 Lys 36 was found not be to affected (Briggs et al. 2002; Ng et al. 2002). These data suggest that Set2 does not play a role in rDNA, telomeric, or mating-type silencing in yeast. In support, preliminary ChIP analyses indicate that H3 Lys 36 methylation is not observed in these regions (data not shown). Additionally, deletion of Set2 does not affect rDNA silencing in Ty1 transposition assays (M. Bryk and F. Winston, pers. comm.). Thus, the role of H3 Lys 36 methylation appears to be distinct from these other sites of methylation. In our effort to further determine the functions of Set2 and H3 Lys 36 methylation, we found an unexpected link with this enzyme, and the transcription apparatus that then uncovered the existence of a pathway selective for H3 Lys 36 methylation. In striking parallel to H2B ubiquitination, CTD phosphorylation by Ctk1 is required for H3 Lys 36 methylation, but not H3 Lys 4 or Lys 79 methylation (Fig. 5A). These results imply that Set2 is recruited directly to chromatin via its interaction with Ctk1-phosphorylated CTD, although

we do not rule out the possibility that alternate mechanisms may exist to regulate Set2 global methylation such as direct phosphorylation of Set2 by Ctk1. Interestingly, comparisons of these two distinct pathways reveal that in each case, these enzymes are present, yet fail to methylate chromatin in the absence of an associated event (i.e., H2B ubiquitination or CTD phosphorylation). Thus, these data suggest a general requirement for the prerequisite of other chromatin-associated events to establish specific histone methylation patterns in yeast. Whether similar mechanisms exist to regulate histone methylation patterns in higher eukaryotes, remains an intriguing question for future study.

A role for Set2 and H3 Lys 36 in transcription elongation

Very little is known about the alterations that affect chromatin structure during transcription elongation. However, numerous reports have shown that RNA pol II is differentially phosphorylated in its CTD (at positions 2 and 5 in the heptapeptide repeat) in response to transcription, and that this phosphorylation correlates with different stages of transcription elongation through chromatin (Kobor and Greenblatt 2002; Orphanides and Reinberg 2002). For example, evidence shows that whereas Ser 5 CTD phosphorylation occurs preferentially in the promoter regions of RNA pol II-transcribed genes, Ser 2 phosphorylation occurs preferentially in the coding regions (Komarnitsky et al. 2000; Cho et al. 2001; Fig. 3). These data suggest differential functions for CTD phosphorylation during the initiation and elongation process. Although several CTD kinases have been identified (Ctk1, Bur1, Kin28, and Srb10), studies indicate that Ctk1 is the major, if not exclusive, Ser 2-specific CTD kinase (Kobor and Greenblatt 2002; Prelich 2002). In agreement, we find that deletion of *CTK1* abolishes all detectable levels of Ser 2 phosphorylation without loss of Ser 5 phosphorylation (data not shown). Therefore, our data strongly suggest that Set2 is recruited to chromatin through selective interaction with Ser 2-phosphorylated CTD. Although Ser 2 phosphorylation has been shown to occur preferentially in the coding region of genes, we note that Ctk1 and H3 Lys 36 methylation are both found in the promoter regions as well (Cho et al. 2001; Fig. 3), suggesting that Ctk1 phosphorylation, albeit at potentially lower levels, functions to load Set2 at an early step in the transcription elongation process.

In addition to a role for Set2 and H3 Lys 36 methylation in transcription elongation, a significant amount of evidence suggests the involvement of other chromatin-associated activities (e.g., HATs and ATP-dependant chromatin remodeling) during this event (Orphanides and Reinberg 2002; Svejstrup 2002). For example, studies have revealed that the elongator complex that copurifies with hyperphosphorylated RNA pol II contains Elp3, a HAT whose enzymatic activity is import for transcription elongation (Svejstrup 2002). Intriguingly, more recent evidence suggests that this complex may not be associated with elongating RNA pol II on chromatin

(Pokholok et al. 2002). Thus, Set2 may be the major histone-modifying activity associated with the transcription apparatus, a result that may indicate an important role for Set2 and H3 Lys 36 methylation in regulating RNA pol II transcription through chromatin. This idea is supported by recent results showing that strains deleted in Set2 are sensitive to 6-Azauracil, a phenotype correlated with transcription elongation defects, and are synthetically growth deficient with other transcription elongation factors (Li et al. 2002, 2003; J. Greenblatt, pers. comm.).

Outside of Set2, additional evidence supports a role for histone methylation in the transcription elongation process. Recently, Set1 methylation was shown to be associated preferentially with the coding regions of genes (Bernstein et al. 2002). Intriguingly, whereas dimethylation of H3 Lys 4 was found to occur in genes whether they were transcriptionally active or repressed (suggesting that di-methylation of Lys 4 defines a gene's potential for transcription), trimethylation of Lys 4 was found to correlate with, and play a role in, the activation of genes (Santos-Rosa et al. 2002). These findings might indicate an important function for Set1 in the elongation process, although its complex has not been associated directly with RNA pol II nor its modification specifically with elongation events. Nevertheless, these data may indicate a common role for histone methylation in the regulation of this aspect of gene transcription, one that is likely mediated through the selective binding of methyllysine-specific factors.

Set2: an activator or repressor?

Recent reports indicate that Set2 is a transcriptional repressor that functions, at least in part, in the basal repression of the *GAL4* gene (Strahl et al. 2002; J. Landry, T. Hesman, J. Min, R.-M. Xu, M. Johnston, and R. Sternglanz, in prep.). However, given that Set2 is likely traveling with hyperphosphorylated RNA pol II during transcription elongation, an activating role for this enzyme could also be implied. Whereas the role of Set2 in elongation remains unclear, we note that Ctk1 has been linked to both positive and negative regulation of gene activity (Patturajan et al. 1998, 1999). Thus, we speculate that Set2 may also be involved in transcription activation as well as repression, depending on the gene context.

In summary, our results show a physical interaction between Set2 and RNA pol II. Whereas a number of proteins have been described to bind to the phosphorylated state of the RNA pol II CTD, the global requirement for Ctk1 and the CTD tail for H3 Lys 36 methylation provides strong evidence that Set2 and H3 Lys 36 methylation is involved in the transcription elongation process. These data also underscore the intimate relationships and genetic requirements necessary to regulate the outcome of specific histone methylation events (Rad6-mediated ubiquitination of H2B for Lys 4/Lys 79 methylation and Ctk1-mediated phosphorylation of the CTD for Lys 36 methylation). Although our data does not elucidate the precise roles of Set2 and H3 Lys 36 methyl-

ation in transcription elongation, understanding their roles presents a challenge for future investigation. Finally, we note that H3 Lys 36 methylation occurs in a broad range of eukaryotic organisms, including humans (van Holde 1989). Thus, we propose that the association of H3 Lys 36 methylation with transcription elongation is likely to be highly conserved.

Material and methods

Yeast strains

Triple-Flag tagging of Set2 was carried out using the *S. cerevisiae* strain AS4, which has been described elsewhere (Gerton et al. 2000). All gene-deletion strains were obtained from Research Genetics and were in the BY4742 background. CTD tail-deletion strains have been described (Nonet et al. 1987).

Construction of 3×Flag-tagged strain

For 3Flag Set2 tagging, we used the p3Flag-KanMX plasmid (Gelbart et al. 2001) as template for generation of a PCR product that introduced three copies of the Flag epitope at the C terminus of the *SET2* gene by homologous recombination. Primers were 5′-GGATTATCATCAGCATCAACAAGGATGTCTTCTCCTCCACCTTCAACATCATCAAGGGAACAAAAGCTGGAG-3′ (forward) and 5′-CTTCCTTTGGGACAGAAAACTGTGTGAAACAAGCCCCAAATATGCATGTCTGGTTAACTATAGGGCGAATTGGGT-3′ (reverse). The underlined bases indicate the sequence that anneals to p3Flag-KanMX plasmid during PCR, whereas the remaining sequence corresponds to the site of insertion at the *SET2* locus.

Set2-3Flag purification

AS4 and AS4 Set2-3Flag cells were grown to O.D. 1.0 in YPD medium. Cell pellets were resuspended in 25 mL of breaking buffer [10 mM Tris-HCl at pH 8.0, 350 mM NaCl, 0.1%NP-40, 10% glycerol, 1 mM PMSF, 2 µg/mL Pepstatin A, 2 µg/mL Leupeptin, 5 µg/mL Aprotinin, 10 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.1% Phosphatase inhibitor cocktail 1 (Sigma P2850)]. To this suspension, 25 mL of glass beads 425–600 µm (Sigma) was added, and cells were disrupted using a BeadBeater (BioSpec) with 10 pulses (30 sec each) at full power with cooling between cycles. The mixture was transferred to 50-mL Falcon tubes and separated from the beads by centrifugation at 1000 xg for 5 min. Lysates were cleared by ultracentrifugation in a SW28 rotor at 100,000*g* for 1 h at 4°C, and 50 µL of pre-equilibrated EZview Red Anti-Flag M2 Affinity Gel (Sigma; F2426) were incubated with 35 mL of WCE (~100–150 mg of protein) for 2 h at 4°C. After incubation, beads were spun down and washed 4× with 25 mL of buffer for 15 min at 4°C. Set2-3Flag was eluted with 50 µg of 3×Flag peptide in a total volume of 100 µL for 1 h at room temperature. Proteins were precipitated with ice-cold acetone at –20°C overnight and pelleted at 16,000*g* for 30 min at 4°C. Protein pellets were speed-vacuum dried and resuspended in 10 µL of 1× SDS–polyacrylamide loading buffer and examined by 8% SDS-PAGE followed by Coomassie staining.

Identification of proteins by mass spectrometry

Coomassie-stained bands of interest were gel excised and subjected to in-gel trypsin digestion in a ProGest automated digester (Genomic Solutions). Products of the trypsin proteolysis reactions were analyzed by matrix-assisted laser-desorption ionization time-of-flight (MALDI–TOF) mass spectrometry on a Reflex III instrument (Bruker Daltonics). Spectra were internally calibrated with trypsin autoproteolytic peaks. Tryptic peptide masses were used to identify the parent protein by the peptide mass fingerprinting approach using the Mascot search engine (Matrix Science). Searches were restricted to *S. cerevisiae* proteins and only statistically significant scores were considered.

Preparation of yeast WCEs and nuclei

Yeast WCEs were prepared essentially as described in Briggs et al. (2001) and only differed in the breaking buffer used for cell disruption (200 mM Tris-HCL at pH 8.0, 320 mM ammonium sulfate, 5 mM MgCl$_2$, 10 mM EGTA at pH 8.0, 20 mM EDTA at pH 8.0, 1 mM dithiothreitol, 20% glycerol). This buffer, which is of high ionic strength, was reported to preferentially extract almost all RNA pol II in the cell (Patturajan et al. 1998). The breaking buffer also contained additional phosphatase inhibitors (10 mM sodium fluoride, 5 mM sodium phosphate, and 1% phosphatase inhibitor cocktail I; Sigma). For nuclei extraction, yeast strains were grown in 200 mL of YPD medium to an O.D.$_{A600}$ between 2.0 and 2.5. Nuclei were extracted as described by Edmondson et al. (1996).

Electrophoresis and immunoblotting

SDS-PAGE and Western blot analyses were performed using procedures and reagents from Amersham Life sciences. Specific antibody detection was made using the ECL+ chemilunescent kit (Amersham Pharmacia Biotech). Rabbit anti-histone modification-specific antibodies were obtained from Upstate Biotechnology Inc. and the dilutions were as follows: 1:3500 for anti-Me(Lys 36)H3, 1:25,000 for anti-Me(Lys 4)H3, and 1:10,000 for anti-Me(Lys 79)H3. Mouse monoclonal anti-Flag antibody (M2; Sigma) was used at 1 µg/mL. Anti-polymerase CTD antibodies 8WG16 (unmodified CTD), H14 (Ser 5 phosphorylation specific), and H5 (Ser 2 phosphorylation specific) were obtained from Covance Inc. and used at dilutions of 1:500, 1:50,000, and 1:1,150, respectively. The IgM H14 and H5 antibodies were detected using HRP-conjugated Donkey anti-mouse IgM at 1:5000 (Jackson ImmunoResearch Laboratories). For WCE analyses (Inputs), typically 5 µL (~50 µg protein) were used. For detection of methylated or acetylated histones, 3–5 µL of nuclei (~100 µg protein) was used.

Immunoprecipitations

For immunoprecipitation experiments involving Set2-3Flag, 300–400 µL of WCE (containing 1 mg of protein) was incubated with 12.5 µL of pre-equilibrated α-Flag affinity beads (M2; Sigma) for 2 h at 4°C. After three washes in extraction buffer, the bead-bound proteins were analyzed by immunoblot analysis using the antibodies indicated at the dilutions described above. For immunoprecipitations involving phosphorylated CTD, WCEs were incubated with H5 or H14 antibody (at a dilution of 1:400) for 1 h at 4°C. For immunoprecipitation, a goat anti-mouse IgM was added (1:400 dilution; Jackson ImmunoResearch Laboratories) followed by 12.5 µL of pre-equilibrated protein G Sepharose beads (Amersham Pharmacia Biotech) for another 1 h at 4°C. Bead-bound proteins were washed and analyzed as described above.

Generation of SET2 and CTK1 expression constructs

Wild-type full-length Set2-Flag bacterial and yeast expression constructs have been described previously (Strahl et al.

2002). The various truncated Set2 constructs [Set2$_{(1–261)}$ and Set2$_{(533–733)}$] used in this study were generated by PCR amplification using Vent DNA polymerase (New England Biolabs) and the Set2-Flag pCal-n expression construct (Stratagene) as template. PCR products contained a C-terminal Flag epitope and were cloned into either the pCal-n or the PN823 yeast expression plasmids. Deletion of the WW domain in Set2 [Set2$_{wwΔ(476–507)}$] was made by PCR mutagenesis using Set2-Flag pCal-n DNA as template following protocols essentially as described in the Ex-Site PCR-Based Site-Directed Mutagenesis kit (Stratagene). Specific protocol modifications included 50 ng of template DNA and the use of Vent DNA polymerase (New England Biolabs). Primers used for the deletion were 5′-CACCCTTCTCAAATC-TATCATTTTGCTGGC-3′ and 5′-TCATCTAAGGTCTTTA TAGTTCAAGG-3′. The resulting Set2$_{wwΔ(476–507)}$Flag sequence in pCal-n was subcloned into PN823 for yeast expression. All constructs were sequenced for accuracy. The *CTK1* ORF was PCR amplified from genomic DNA using Vent DNA polymerase (New England Biolabs) and cloned into the PN823 plasmid to produce PN823CTK1. This plasmid was transformed into *ctk1Δ* and selected on SC-ura plates. Forward and reverse primer sequences used were 5′-GCGCGGATCCATGTCCTACAAT AATGGCAATACTTATTC-3′ and 5′-CGCGTCGACTTATT TATCATCATCGTCATTATTATTATTATTATTATTAC TATTACC-3′.

RT–PCR

For RT–PCR, the yeast strains indicated in the figures were grown in 100 mL of YPD to an O.D.$_{600}$ of 1.0. Total RNA was isolated using the Clontech Nucleospin RNA Purification Kit and 100 µg of total RNA was subjected to polyA+ messenger RNA isolation using the QIAGEN Oligotex mRNA Kit. The resulting mRNA was used to synthesize complimentary DNA using M-MLV Reverse Transcriptase (Invitrogen) as per the manufacturer's directions. The PCR reaction consisted of 1/20 of the cDNA collected from the room-temperatuture reaction and primers specific to the indicated genes. Primers for the *SET2* and ACT1 ORFs encompassed positions +1940 to +2202 and +1263 to +1416 relative to the transcription initiation site, respectively. Negative controls using no room temperature were also included.

ChIP assay

The chromatin immunoprecipitation assay was performed as described previously (Kuo and Allis 1999). Briefly, WCEs were prepared from formaldehyde-fixed wild-type and *set2Δ* cells (grown to O.D.$_{600}$ 1.0) and sonicated to shear their chromatin. In these assays, chromatin was sonicated to an average of 350 bp with a size range of 250–500 bp. This was followed by immunoprecipitation using Protein A Sepharose (Amersham Pharmacia Biotech) or anti-mouse IgM Agarose (ICN) with the following antibodies at 1 µL/IP: α-Me(Lys 4)H3, α-Me(Lys 36)H3, 8WG16, H14 and α-Ctk1 phosphorylated CTD (Lee and Greenleaf 1991). After cross-link reversal, DNA was extracted for PCR. For all genes tested, we designed primers to amplify promoter regions that were approximately either –400 to –200 bp or –200 to –30 bp relative to the translation start-site and coding-region sequences corresponding to approximately the middle of the ORF or last 200 bp 5′ to the stop codon. The PCR amplification reactions used Taq DNA polymerase (Promega) and consisted of 1/50 of the precipitated DNA along with the appropriate promoter or coding-region primers. Products were analyzed on a 1.5% agarose gel. The following fragments of selected genes were used to design primers for promoter and transcribed re-

gions: *PMA1* promoter, positions –623 to –390 and –393 to –98; *PMA1* ORF, positions +1266 to +1437 and +2347 to +2520; *GSY2* promoter, positions –479 to –272 and –274 to –44; *GSY2* ORF, positions +895 to +1070 and +1821 to +1989; *GAL4* promoter, positions –462 to –258 and –268 to –41; *GAL4* ORF, positions +1195 to +1369 and +2351 to +2516; *GAL10* promoter, positions –509 to –271 and –274 to –37; *GAL10* ORF, positions +903 to +1079 and +1790 to +1975. All positions are relative to the translation initiation site.

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