The Paf1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p*

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Monoubiquitination of histone H2B, catalyzed by Rad6-Bre1, is required for methylation of histone H3 on lysines 4 and 79, catalyzed by the Set1-containing complex COMPASS and Dot1p, respectively. The Paf1 protein complex, which associates with RNA polymerase II, is known to be required for these histone H3 methylation events. During the early elongation stage of transcription, the Paf1 complex is required for association of COMPASS with RNA polymerase II, but the role the Paf1 complex plays at the promoter has not been clear. We present evidence that the Paf1 complex is required for monoubiquitination of histone H2B at promoters. Strains deleted for several components of the Paf1 complex are defective in monoubiquitination of histone H2B, which results in the loss of methylation of lysines 4 and 79 of histone H3. We also show that Paf1 complex is required for the interaction of Rad6 and COMPASS with RNA polymerase II. Finally, we show that the Paf1 complex is required for Rad6-Bre1 catalytic activity but not for the recruitment of Rad6-Bre1 to promoters. Thus, in addition to its role during the elongation phase of transcription, the Paf1 complex appears to activate the function but not the placement of the Rad6-Bre1 ubiquitin-protein ligase at the promoters of active genes.

The DNA of eukaryotic organisms assembles around histone proteins to form highly organized structures known as chromatin (1). Alterations in chromatin structure play a major role in regulating gene expression, and for this reason much attention has been focused recently on the covalent modifications of histone proteins and their outcomes in transcriptional elongation (1–4). Essential to this process are the N-terminal tails of histone proteins. Because they protrude from the globular body of the nucleosome and are available for interactions with other proteins, the tails are the site of many covalent modifications that alter nucleosome structure. A myriad of modifications, such as acetylation, phosphorylation, ubiquitination, and methylation, decorate each histone tail (2, 5) The combinatorial effects of such modifications can produce an array of different responses involved in transcriptional activation and repression (1–6).

One histone modification of major consequence is the methylation of histone H3 at lysines 4 and 79, catalyzed by the Set1-containing complex COMPASS and Dot1p, respectively (7–14). It has been shown that methylation of both lysine residues impacts the expression of genes within the rDNA loci and telomeric regions of DNA in Saccharomyces cerevisiae (7–8, 15, 16). It has been demonstrated that some of the components of the Paf1 complex, a complex that associates with the initiating and elongating RNA polymerase II, is also required for histone H3 methylation on lysines 4 and 79 (17, 18). Accordingly, previous studies have demonstrated a role for the Paf1 complex in transcriptional elongation and initiation (19–22). A further requirement for the methylation of both lysines 4 and 79 of histone H3 is the prior modification of another histone protein; monoubiquitination of histone H2B, catalyzed by the Rad6-Bre1 protein-ubiquitin ligase (23–28). Although the Paf1 complex also plays a role in histone H3 Lys-36 methylation by Set2, this modification does not require monoubiquitination of histone H2B. Because the Paf1 complex is associated with RNA polymerase II at the promoter, we wished to determine whether it is also required for the monoubiquitination of histone H2B.

Here we present evidence that components of the Paf1 complex are required for monoubiquitination of histone H2B. Strains in which several components of the Paf1 complex are deleted are defective in monoubiquitination of histone H2B at promoter regions, and this in turn results in the loss of methylation on histone H3 on lysines 4 and 79 within the body of active genes. Previously we demonstrated that the Paf1 complex is required for the interaction between RNA polymerase II and COMPASS, and that loss of the Paf1 subunit Ctr9 dissolved this interaction (17). We have recently found that Rad6 associates with RNA Pol II and COMPASS, and the Paf1 complex mediates this interaction as well. However, chromatin immunoprecipitation (ChIP) experiments indicate that the Paf1 complex is not required for the recruitment of Rad6-Bre1 to the promoter. Together, our studies indicate a role for the Paf1 complex at the promoter in regulating the functional activity of the Rad6-Bre1 complex in monoubiquitination of histone H2B, which is distinct from the role of the Paf1 complex in histone methylation during the process of transcription elongation.

The abbreviations used are: COMPASS, complex of proteins associated with Set1; Pol II, polymerase II; E3, ubiquitin-protein ligase; ChIP, chromatin immunoprecipitation; TAP, tandem affinity purification; NIB, nuclear isolation buffer.
Pelleted by centrifugation at 2000 rpm. Cells were then resuspended in 500 μl of NIB (0.25 M sucrose, 60 mM KCl, 14 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 0.8% Triton X-100), and then pelleted again. After removal of the supernatant by pipetting, cells were incubated on ice for 30 min with occasional vortexing. Cellular debris were removed by centrifugation at 15,000 rpm, the supernatants were pelleted by centrifugation at 2000 rpm, and the pellets were resuspended in 75 μl of sterile water and 25 μl of Laemmli loading buffer. Extracts were then heated at 95°C for 5 min and resolved using 16% SDS-PAGE. The extracts were then transferred to a fresh 1.5-ml tube, acetone was added at a 1:5 ratio (1 ml in this case), and the tubes were kept on ice for 45 min. Free histones were pelleted by centrifugation at 15,000 rpm, the supernatants were drained, and the pellets were resuspended in 75 μl of sterile water and 25 μl of Laemmli loading buffer. Extracts were then heated at 95°C for 5 min and resolved using 16% SDS-PAGE.

**FIG. 1.** Localization of Rad6 to the promoter region of a gene requires the E3 ligase Bre1. A, to examine the recruitment of Rad6 to different regions of the PMA1 gene in a Rad6-TAP strain, ChIP was performed in triplicate using PCR primers directed against the promoter (set 1), early body (set 2), middle (set 3), and late body (set 4) of the gene. Each PCR reaction also contains a control primer set. The fold enrichment is determined as the ratio of experimental to control signal for the immunoprecipitated DNA divided by the ratio of the experimental to control signal for the input DNA. Heat6 localization is also examined in a Rad6-TAP strain in which BRE1 has been deleted. Rad6 localizes to the promoter region of PMA1 (dark gray bars), and the loss of Bre1 abolishes Rad6 recruitment (light gray bars). B, TAP::Bre1, which does not interact with Rad6, localizes to the promoter region of PMA1 indicating that the interaction of Bre1 with the promoters is independent of its interaction with Rad6. Once again, ChIP was performed in triplicate as described in A. C, illustration of the four sets of primers used for ChIP.

**MATERIALS AND METHODS**

**Preparation of Histone Extracts**—Histone extraction was performed as described in (31) with several changes. 50 ml of YPD (yeast peptone/dextrose) were inoculated and allowed to grow at 30°C to an A₆₀₀ of ~1. The cultures were then transferred to 50-ml conical tubes and the cells pelleted by centrifugation at 2000 rpm. Cells were then resuspended in 500 μl of sterile water and transferred to 1.5-ml Eppendorf tubes (cell pellet size ~150–200 μl). Cells were then pelleted by centrifugation at 2000 rpm, resuspended in 500 μl of NIB (0.25 M sucrose, 60 mM KCl, 14 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 0.8% Triton X-100), and then pelleted again. After removal of the supernatant by pipetting, cells were resuspended in another 500 μl of NIB, and ~150 μl of 0.5-mm glass beads were added to one-third of the total volume of the suspension. The tubes were then closed, and lids were wrapped in parafilm to prevent leakage during lysis. The tubes were then vortexed vigorously for 15 min at 4°C, and lysates were recovered into 15-ml conical tubes by puncturing the bottom of the 1.5-ml tube with a small-gauge needle, inserting the punctured tube into the top of the 15-ml tube, and briefly centrifuging the contents at 2000 rpm. The supernatants were discarded, and cell pellets were washed with 1 ml of chilled NIB followed by centrifugation to pellet the nuclei. After the supernatant was discarded, the nuclei were suspended in 200 μl of 0.4 N H₂SO₄ and incubated on ice for 30 min with occasional vortexing. Cellular debris were then pelleted by centrifugation at 2000 rpm. The supernatants were transferred to a fresh 1.5-ml tube, acetone was added at a 1:5 ratio (1 ml in this case), and the tubes were kept on ice for 45 min. Free histones were pelleted by centrifugation at 15,000 rpm, the supernatants were drained, and the pellets were resuspended in 75 μl of sterile water and 25 μl of Laemmli loading buffer. Extracts were then heated at 95°C for 5 min and resolved using 16% SDS-PAGE.

**ChIP Experiments**—Overnight culture of cells were treated with formaldehyde to achieve cross-linking as described previously (26). After the cross-linked cells were lysed, the chromatin was isolated and sheared by sonication. The sheared chromatin solution was then subjected to immunoprecipitation using IgG-Sepharose beads. Immunoprecipitates were eluted and de-cross-linked, and then the free DNA was precipitated and used for PCR with primer pairs directed against different regions of the PMA1 gene. Each reaction contains a control primer set directed against a region of yeast chromosome 5 with no open reading frames. The fold enrichment was determined by comparing the ratio of the immunoprecipitated PCR product to input DNA PCR product using ImageQuant software (Amersham Biosciences).

**RESULTS AND DISCUSSION**

**Bre1 Is Required for Recruitment of Rad6 to the Promoter—**Rad6 is required for monoubiquitination of histone H2B on lysine...
extracts were prepared to demonstrate that the tagging of Cps60/Bre2 is dispensable for its localization to promoters (Fig. 1).

Both the components of the Paf1 complex and the ubiquitination machinery at the promoter, suggesting that (a) Bre1 is the protein that targets Rad6 to promoters and (b) the C-terminal domain of Bre1 is dispensable for its localization to promoters (Fig. 1).

**Paf1 Complex Affects Both Histone H3 Methylation and H2B Ubiquitination**—Both the components of the Paf1 complex and the ubiquitin activity of the Rad6-Bre1 complex are required for methylation of histone H3 on both lysines 4 and 79 (Fig. 2A) (24–27). To determine whether the Paf1 complex is also required for histone H2B ubiquitination via Rad6-Bre1, we tested whether H2B is ubiquitinated in the absence of either of the Paf1 complex subunits Rtf1 or Paf1. Interestingly, deletion of either *RTF1* or *PAF1* resulted in the loss of histone H2B ubiquitination (Fig. 2B). Ubiquitination of histone H2B and methylation of histone H3 were complemented by introducing a plasmid containing either wild-type *RTF1* or *PAF1* into the mutant cells lacking Rtf1 or Paf1 (Fig. 2C).

**Interaction among Rad6, COMPASS, and RNA Polymerase II Requires Paf1 Complex**—COMPASS interacts with RNA polymerase II during the early stages of transcriptional elongation (17–18). To determine whether there are interactions between COMPASS, RNA polymerase II, and Rad6, COMPASS was purified via one of its tagged subunits, the Ash2 homologue CPS60/Bre2. RNA polymerase II co-purifies with COMPASS, as does Rad6 (Fig. 3, A–C, lanes 6 and 7), indicating that a functional as well as physical interaction exists between the ubiquitination and methylation machinery at the promoter region of active genes. However, in strains missing the gene encoding the Rtf1 subunit of the Paf1 complex, Rad6 no longer associates with COMPASS and RNA polymerase II (Fig. 3C, lanes 8 and 9), but the interaction between COMPASS and Pol II remains stable (Fig. 3, A and B, lanes 8 and 9). From this it seems likely that the deletion of Rtf1 does not affect the stability of the Paf1 complex but may instead be a part of the site where Rad6 interacts.

Previously, we demonstrated that the Ctr9 subunit (one of the largest subunit) of the Paf1 complex is required for the interaction of COMPASS and RNA polymerase II (17). However, removal of the Rtf1 component of the Paf1 complex has no effect on the interaction between RNA polymerase II and COMPASS (Fig. 3, A and B, lanes 8 and 9), indicating that COMPASS may physically interact with Ctr9 subunit of the Paf1 complex or that Ctr9 is essential for the stability of the Paf1 complex and therefore its interaction with RNA polymerase II. We have also demonstrated that the interaction between RNA polymerase II and COMPASS with Rad6 is dependent on the presence of the Rtf1 subunit of the Paf1 complex (Fig. 3C, lanes 6–9).

**Paf1 Complex Is Not Essential for Rad6 Recruitment to the Promoter**—A simple model that could account for these observations is that the Paf1 complex is responsible for the localization of the Rad6-Bre1 complex to the promoter, possibly by acting as a recruitment platform for the ubiquitination machinery to bind. To determine whether the Paf1 complex is indeed required for Rad6-Bre1 recruitment to promoters, ChIP expen-
Paf1 Is Essential for Histone Monoubiquitination by Rad6-Bre1

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FIG. 5. Model demonstrating the role of the Paf1 complex in the functional activation of the Rad6-Bre1 complex in ubiquitination of histone H2B at promoters. A, the E2 ubiquitin ligase Rad6 is recruited to the promoter via its E3 ligase Bre1 (26). After the assembly of the preinitiation complex, the C-terminal domain (CTD) of RNA Pol II is phosphorylated on serine 5 by Kin28. B, the Paf1 complex (Paf1c) enters the preinitiation complex and mediates the interaction among Rad6-Bre1, COMPASS, and RNA Pol II. This interaction is required for the functional activation of Rad6-Bre1 in ubiquitination of histone H2B on lysine 123. C, after the promoter region is ubiquitinated by Rad6-Bre1, the complex of RNA Pol II, COMPASS, and the Paf1c enters early elongation as COMPASS methylates Lys-4 of histone H3 in the early body of the gene.

Fig. 5. Model demonstrating the role of the Paf1 complex in the functional activation of the Rad6-Bre1 complex in ubiquitination of histone H2B at promoters.