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Bre1, an E3 Ubiquitin Ligase Required for Recruitment and Substrate Selection of Rad6 at a Promoter

Short Article

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Summary

Ubiquitination of histone H2B catalyzed by Rad6 is required for methylation of histone H3 by COMPASS. We identified Bre1 as the probable E3 for Rad6's role in transcription. Bre1 contains a C3HC4 (RING) finger and is present with Rad6 in a complex. The RING finger of Bre1 is required for ubiquitination of histone H2B, methylation of lysine 4 and 79 of H3 and for telomeric silencing. Chromatin immunoprecipitation experiments indicated that both Rad6 and Bre1 are recruited to a promoter. Bre1 is essential for this recruitment of Rad6 and is dedicated to the transcriptional pathway of Rad6. These results suggest that Bre1 is the likely E3 enzyme that directs Rad6 to modify chromatin and ultimately to affect gene expression.

Introduction

Covalent modifications of the histone proteins in nucleosomes are important for proper regulation of gene expression (Workman and Kingston, 1998; Urnov and Wolffe, 2001; Wolffe, 2001). Methylation of histone H3 on lysine 4 catalyzed by COMPASS (a protein complex that includes Set1) (Krogan et al., 2002a; Miller et al., 2001; Roguev et al., 2001; Nagy et al., 2002) and on lysine 79 (catalyzed by the Dot1 protein) (van Leeuwen et al., 2002; Ng et al., 2002a, 2002b) is required for silencing of expression of genes located near the telomeres of chromosomes in *S. cerevisiae*. For histone H3 to be methylated by COMPASS and Dot1, histone H2B must be ubiquitinated on lysine 123, and this is catalyzed by the Rad6 ubiquitin-conjugating enzyme (Dover et al., 2002; Sun and Allis, 2002; Ng et al., 2002b; Robzyk et al., 2000). Since Rad6 is involved in diverse biological processes (Jentsch et al., 1987; Koken et al., 1991; Bailly et al., 1994; Hishida et al., 2002; Kupiec and Simchen, 1986; Kang et al., 1992; Huang et al., 1997), different ubiquitin-protein isopeptide ligases (E3 enzymes) are presumably responsible for specifying its different activities. Thus, the E3 enzyme that directs Rad6 to ubiquitinate histone H2B is expected to be a key determinant of transcriptional regulation.

In this manuscript, we identify Bre1 as a RING finger-containing protein that is: (1) found in a macromolecular complex with Rad6; (2) required for the ubiquitination of histone H2B in vivo, which seems to be a signal for methylation of histone H3 at its fourth and seventy-ninth lysines; (3) required for telomeric silencing; (4) recruited with Rad6 to a promoter; (5) essential for the recruitment of Rad6 to chromatin at a promoter; and (6) dedicated to the transcriptional regulatory role of Rad6. Taken together, the data presented in this study strongly suggest that Bre1 is the likely E3 ubiquitin ligase for Rad6 in its role in regulating chromatin structure and transcription.

Results and Discussion

Bre1 Is Required for the Proper Histone H3 Methylation by COMPASS

To identify the E3 enzyme that directs Rad6 to modify histone H2B, we tested each of the ~4800 nonessential yeast gene deletion mutants for methylation of lysine 4 of histone H3. We discovered that *BRE1* (*YDL074c*) is required for the methylation of the fourth lysine of histone H3 (Figure 1A) as described in the Experimental Procedures. *BRE1* was initially identified based on hypersensitivity of the *bre1* mutants to brefeldin A, a drug that affects intracellular protein transport (Oyen et al., 2001). Bre1 contains near its C terminus a C3HC4 (RING) zinc finger (Figure 1B), a motif found in several other proteins, including Rad18, Rad16, Rad5, BRCA1, and Snf2L (Figure 1B) (Conaway et al., 2002; Hochstrasser, 2000; Ulrich and Jentsch, 2000). RING finger proteins contain a characteristic C3HC4 or C3H2C3 motif (Figure 1C) that binds a zinc ion and appear to act as E3 enzymes that play important roles in many processes, including transcriptional regulation, cell cycle progression, oncogenesis, signal transduction, and development (Kamura et al., 1999; Swanson et al., 2001; Lu et al., 2002). Two independently generated *bre1* mutants were found to be defective for K4 methylation of histone H3 (Figure 1D). Methylation of H3 was restored by introduction into these strains of a plasmid containing wild-type *BRE1* but not a plasmid carrying point mutations affecting key amino acids of the RING finger domain of Bre1 (Figure 1E).

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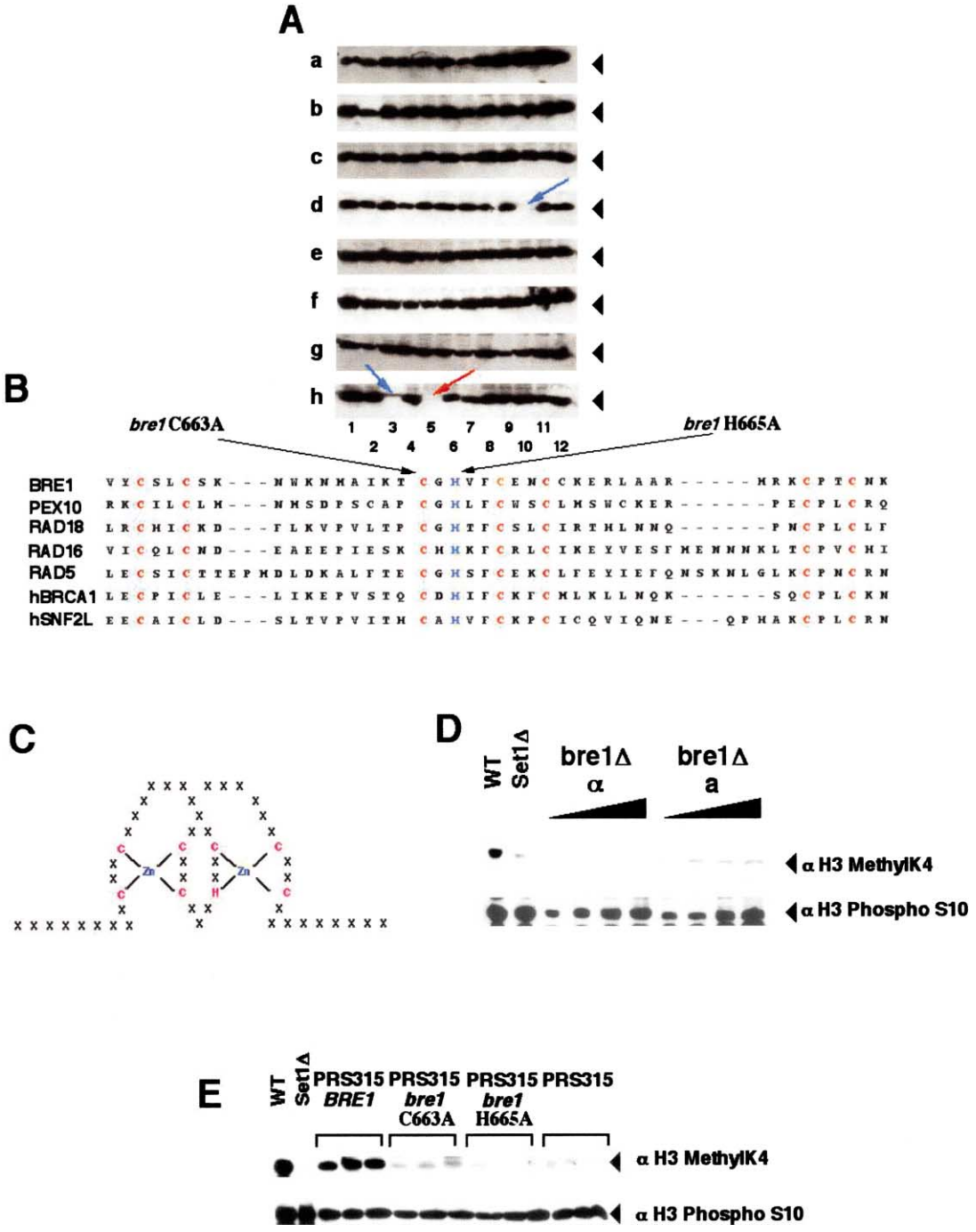

Figure 1. Surveying the *S. Cerevisiae* Genome Identified Bre1, a C3HC4 (RING) Finger-Containing Protein, as a Gene Required for Methylation of Lysine 4 of Histone H3

(A) Extracts of *S. cerevisiae* mutants missing one of the approximately 4800 nonessential genes (Dover et al., 2002) were tested for the presence of Lys4-methylated histone H3. One of the mutants lacking this histone modification is *bre1* (row H, lane 5). Arrows at position d10 and h3 indicate empty wells as plate markers.

(B) Sequence comparison of several other proteins containing a C3HC4 domain.

(C) Structure of a C3HC4 (RING) finger domain.

(D) Extracts of wild-type strains deleted for *set1* or two independently generated mutant strains (MATa and MATα) deleted for *bre1* were tested for the presence of Lys4-methylated histone H3. The presence of histone H3 phosphorylated at its serine 10 was used as an indicator of equivalent loading.

(E) The K4 methylation-deficient phenotype of the *bre1*Δ cells was complemented by either an episomal vector containing either full-length cDNA for Bre1 or point mutated *bre1* (C703S) or (H705A).

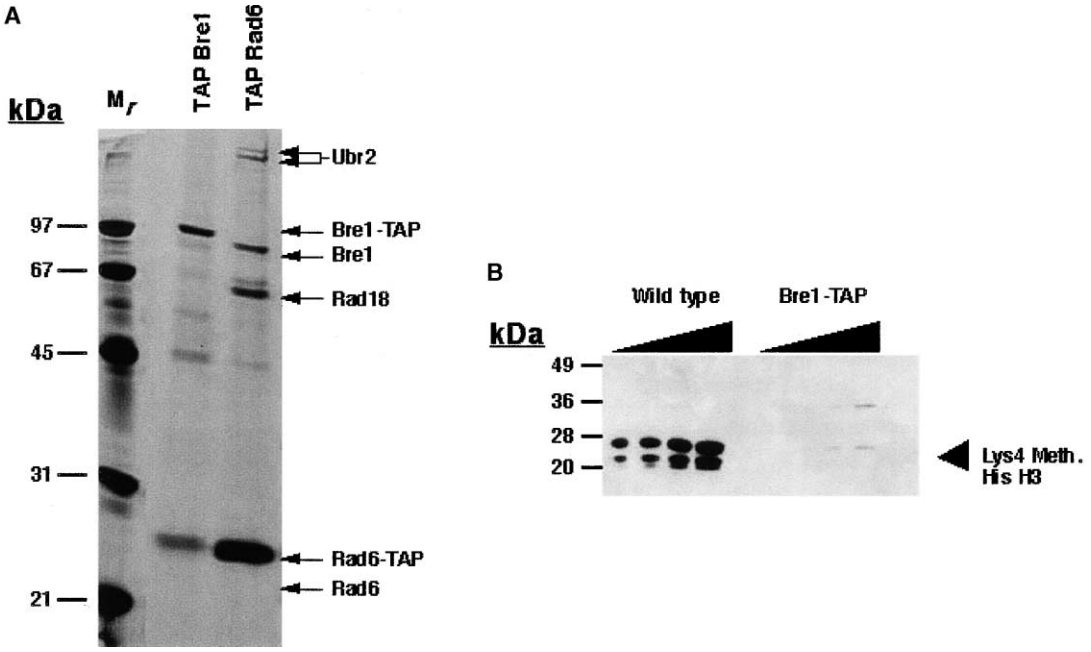

Figure 2. Biochemical Characterization of the Bre1- and Rad6-Containing Complexes

(A) Tandem affinity purification (TAP) of Bre1 and Rad6. Silver stain analysis of TAP-tag affinity-purified Bre1 and Rad6 complex. Tagged-Rad6 copurified with Bre1, Rad18, Ubr1, and Ubr2. The position of tagged and untagged Bre1 and Rad6 is indicated by the arrow.

(B) Histone methylation at K4 H3 was tested in either wild-type or Bre1-TAP strains.

The Ring Finger of Bre1 Is Required for Its Molecular Function In Vivo

To identify proteins that physically associate with Bre1, we attached an affinity tag (TAP) to the C termini of Bre1 and Rad6 and purified them by affinity purification as described in the Experimental Procedures. Rad6-TAP copurifies with Bre1, Rad18, Ubr1, and Ubr2. However, Bre1-TAP was isolated as a single subunit with no trace of Rad6 (we were even unable to detect Rad6 by Western blot analysis using polyclonal antibodies specific to Rad6 [data not shown]). Since the C3HC4 RING finger domain is very close to the C terminus of Bre1, it seemed possible that the TAP affinity tag attached to the C terminus of Bre1 disrupts its function. Indeed, the strain with the chimeric Bre1 is defective in methylation of K4 of histone H3 (Figure 2B), suggesting that C3HC4 RING finger domain of Bre1 is required for interaction with Rad6. When the purified Rad6-containing complex is subjected to size exclusion chromatography, Rad6 comigrates with Bre1, Rad18, Ubr1, and Ubr2 (data not shown). This observation is consistent with the idea that Bre1 associates with Rad6. These experiments indicate that Rad6 and Bre1 can physically interact and that the RING finger of Bre1 is required for this biochemical interaction.

Bre1 Is Required for Monoubiquitination of Histone H2B In Vivo

Rad6-catalyzed ubiquitination of histone H2B (Robzyk et al., 2000) is required for efficient methylation of lysine 4 and lysine 79 of histone H3 (Dover et al., 2002; Sun and Allis, 2002; Briggs et al., 2002; Ng et al., 2002b). Therefore, we tested whether Bre1 is involved in the ubiquitination of histone H2B. In a strain whose only copy of the H2B gene is fused to the FLAG epitope tag, the ubiquitinated histone H2B can be differentiated from the unmodified form by SDS-PAGE and Western blot analysis using either an antibody against the Flag epitope or anti-ubiquitin specific antibodies (Figure 3A, lanes 1–3); only the more slowly migrating form of H2B is detected by anti-ubiquitin antibodies (Figure 3A, lower panel, lanes 1–3). Deletion of *RAD6* or *BRE1* from this strain results in the loss of the slowly migrating, ubiquitinated H2B (Figure 3A, lanes 4–9).

Bre1 Is Essential for Methylation of Histone H3 on Lysine 4 and 79 and Is Dedicated to the Transcriptional Role of Rad6

Since ubiquitination of K123 of H2B (Figure 3A) is required for methylation of K79 of H3, we tested whether Bre1 is also required for this modification of histone H3. Indeed, a strain lacking *BRE1* has significantly reduced levels of lysine 79-methylated histone H3 (Figure 3B), indicating that Bre1 plays the same role in histone modifications as does Rad6.

If Bre1 is indeed the E3 enzyme for Rad6, it should also be required for silencing of genes located near chromosome telomeres (van Leeuwen et al., 2002; Krogan et al., 2002a; Dover et al., 2002; Huang et al., 1997). Indeed, *BRE1*, like *RAD6*, appears to be required for normal silencing of a *URA3* gene located near the telomere of chromosome VII (Figure 3C). However, unlike *rad6* mutants, a *bre1* mutant is not as sensitive to DNA-damaging agents such as UV irradiation (Heude et al., 1995) (Figure 3F), or to MMS (Ulrich, 2001; Ramotar and Masson, 1996) (Figure 3D) and to phleomycin (He et al.,

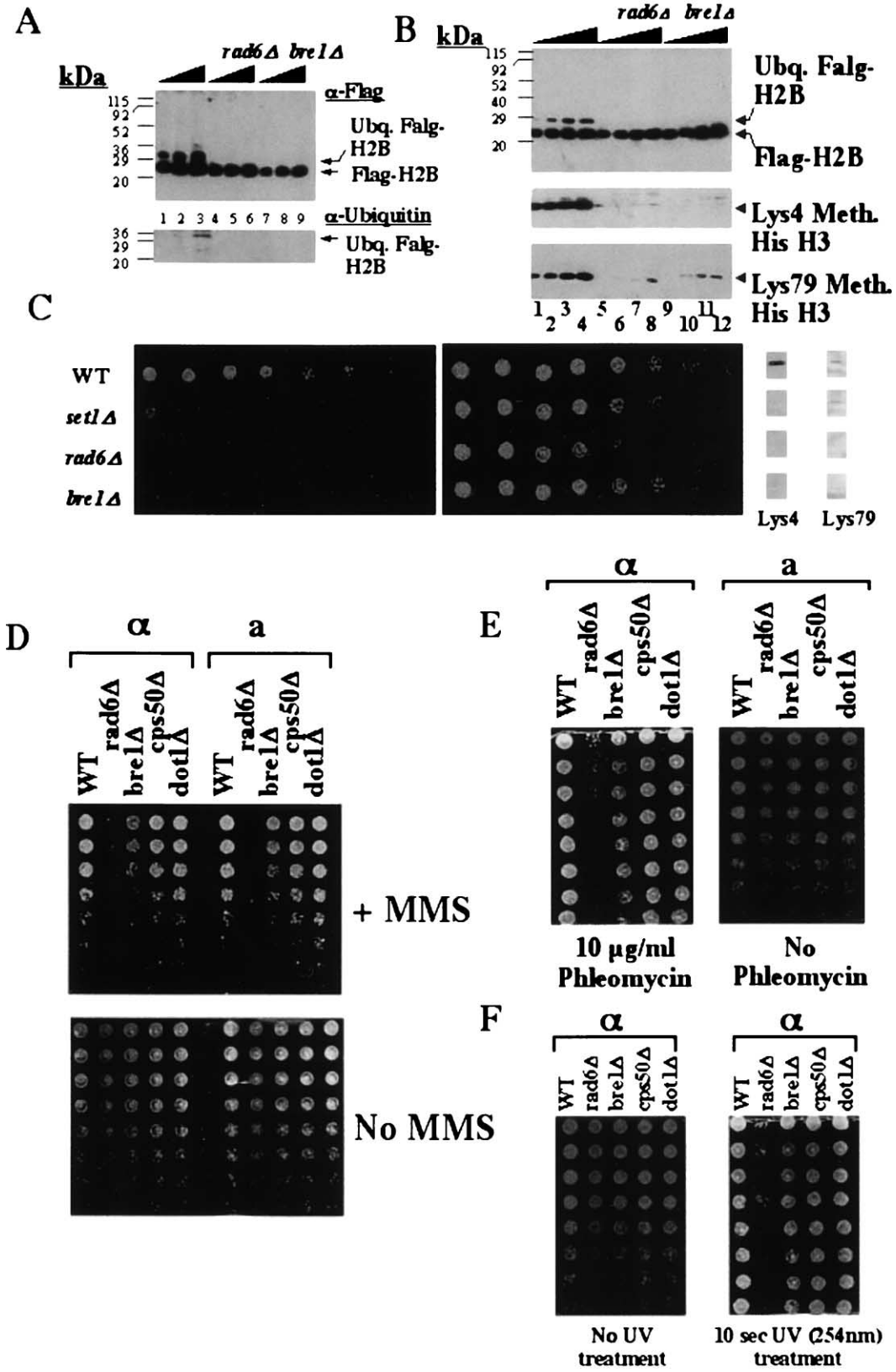

Figure 3. The Role of Bre1 in Histone H2B Ubiquitination, Histone H3 Lysine 79 Methylation, and Telomeric Silencing

(A) Bre1 is required for the ubiquitination of histone H2B. Increasing concentrations of highly purified acid extracted histones from wild-type strains (strains containing FLAG-tagged H2B as the only source of histone H2B) or strains deleted for Rad6 or Bre1 in the same background, were analyzed by Western blotting using (upper panel) monoclonal antibodies against the FLAG epitope or (lower panel) polyclonal antibody

1996) (Figure 3E). Thus, Bre1 seems to be dedicated to the transcriptional regulatory function of Rad6.

Bre1 Is Recruited to a Promoter and Is Required for the Recruitment of Rad6 to a Promoter

To further determine the role of Bre1 as the ligase for Rad6 in chromatin modification by histone ubiquitination, we employed chromatin immunoprecipitation (ChIP) as described in the Experimental Procedures. We have used *PMA1* gene for these studies because it is a highly transcribed gene. Our analysis demonstrated that Rad6 is directed toward chromatin specifically at the promoter (Figure 4A). To determine whether Bre1 can direct Rad6 toward the promoter, ChIP experiment for Rad6 was performed in cells lacking Bre1. As seen in Figure 4B, Bre1 is essential for the recruitment of Rad6 to chromatin at a promoter, further supporting a role of Bre1 as the E3 enzyme for Rad6 in transcription.

Recent evidence suggests that ubiquitination of histone H2B on its lysine 123, catalyzed by Rad6, directs COMPASS and Dot1 to methylate Lys4 and Lys79 of histone H3. Since Rad6 (E2) is involved in diverse biological processes such as DNA repair, DNA damage-induced mutagenesis, meiosis, transposition of retrotransposons, and gene silencing (Jentsch et al., 1987; Koken et al., 1991; Bailly et al., 1994; Hishida et al., 2002; Kupiec and Simchen, 1986; Kang et al., 1992; Huang et al., 1997) different ubiquitin-protein isopeptide ligases (E3 enzymes) likely determine the specificity of Rad6 and regulate its function. Several pieces of evidence reported here suggest that Bre1 is the E3 enzyme that directs Rad6 to catalyze ubiquitination of lysine 123 of histone H2B. First, Bre1, like Rad6, is required for ubiquitination of H2B (Figure 3A). Second, Bre1 possesses a protein motif—the RING finger—characteristic of E3 enzymes. Third, Bre1 physically associates with Rad6 (Figure 2). Other proteins that associate with Rad6, such as Rad18 (which also contains a RING finger), Ubr1, and Ubr2, are not required for methylation of H3 and therefore for ubiquitination of H2B and are unlikely to be the E3 enzymes for Rad6 in this pathway (Dover et al., 2002). Two other proteins that have been functionally linked to Rad6—Rex4 and Ubr1—are also dispensable for ubiquitination of H2B (Dover et al., 2002). Fourth, both Rad6 and Bre1 are recruited to chromatin at a promoter. Fifth, Bre1 is essential for the recruitment of Rad6 to chromatin at a promoter. Finally, Bre1 seems to be dedicated to the transcriptional regulatory role of Rad6. Our results, taken together, suggest that Bre1 is the E3 enzyme that directs Rad6 to its role in regulating chromatin structure and transcription.

We are unable to detect autoubiquitination of recombinant Rad6 with Bre1 or even with its known E3 ligases Ubr1/2 or Rad18. Autoubiquitination is an intrinsic property of some E2s but not of others, and it appears that Rad6 falls in the class of E2s that cannot be activated to autoubiquitinate. Since we find that Rad6 is recruited to nucleosomes at a promoter via Bre1 but not to the body of the gene, we believe Rad6 must be recognizing something that is special about nucleosomes at the promoters, such as acetylation, phosphorylation, or other modifications. For this reason, defining the nucleosome substrate of Rad6-Bre1 is difficult to isolate at this time.

A fundamental role for protein ubiquitin in the regulation of transcription via RNA polymerase II is rapidly becoming clear (Conaway et al., 2002). Identification of the E3 enzymes involved in the regulation of transcription by ubiquitination is essential for understanding the specificity and regulation of any given pathway. Identification of Bre1 as the likely E3 enzyme that links Rad6 to the transcription machinery has now opened the door for the molecular dissection of the role of histone H2B ubiquitination and transcriptional regulation.

Experimental Procedures

Functional Genomic Analyses of Histone Modification by Methylation

Using a 96-well pinning device, the entire collection of 4800 yeast nonessential gene deletion mutants were inoculated from −80°C stocks onto agar plates containing YPD + 200 μg/ml GENETICIN (GIBCO) and allowed to grow 48 hr and used to inoculate 96-tube PCR-plates filled with 100 μl of YPD. After 48 hr of growth at 30°C, the plates were centrifuged at 2000 g for 10 min. The medium was removed by wrist-snap inversion and drained into absorbent towels. The plates were then covered and frozen at −80°C for up to 1 week. Cells were thawed at room temperature, resuspended in 30 μl lysis buffer (20 mM Tris [pH 7.5], 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1%NP40, 1 mg/ml Zymolyase 100 T), and incubated at 37°C for 15 min. Ten microliters of 4× Laemmli loading buffer was added, and the samples were vortexed briefly before heating at 100°C for 5 min. The lysates were subjected to SDS-PAGE, transferred to

raised against ubiquitin. As indicated by the arrows, Ubq.-H2B is the slower migrating form, and the unubiquitinated species of histone H2B is the faster migrating form.

(B) To determine the role of Bre1 in methylation of K79 of histone H3, extracts of wild-type strains or strains deleted for *rad6* and *bre1* were tested for the presence of ubiquitinated H2B and methylated K4 and K79 of histone H3.

(C) Deletion of Bre1 results in a defect in silencing of gene expression at telomeres. Either the wild-type parental strain (UCC1001, harboring *URA3* near the left telomere of chromosome 7 as a reporter of telomeric gene silencing [Nislow et al., 1997]) or UCC1001 deleted for *set1*, *rad6*, or *bre1* was tested for defect in silencing of gene expression at telomeres. Wild-type cells silence expression of the telomere-associated *URA3* gene and are therefore resistant to 5FOA. Cells defective for telomeric gene silencing have increased expression of *URA3* and hence are sensitive to 5FOA (Nislow et al., 1997). Two-fold serial dilutions of cultures (from about 5×10^4 to 50 cells) were spotted on minimal glucose plates containing (left panel) or lacking (right panel) 5FOA. These plates were incubated at 30°C for 36 (±4) hr. (Far right panel) The ability of each strain to methylate its histone H3 either on K4 or K79 was tested by Western analysis. In the same experiment, the addition of plasmid containing wild-type Bre1 and not the (RING) finger-mutated *bre1* complemented the silencing phenotype of bre1Δ strains (data not shown).

(D–F) To inquire whether Bre1 is dedicated to Rad6's role in histone ubiquitination or is involved in some of the other functions in which Rad6 plays a role, we tested whether bre1 mutant is as sensitive to DNA damaging agents such as UV irradiation (254 nM) or to chemicals treatment such as MMS or phleomycin. *Rad6* mutant cells have been demonstrated to be sensitive to such treatments. As a control, we also tested the sensitivity of yeast cells deleted for either *cps50* subunit of COMPASS (involved in histone H3 Lys4 methylation) or *dot1* (involved in histone H3 Lys79 methylation).

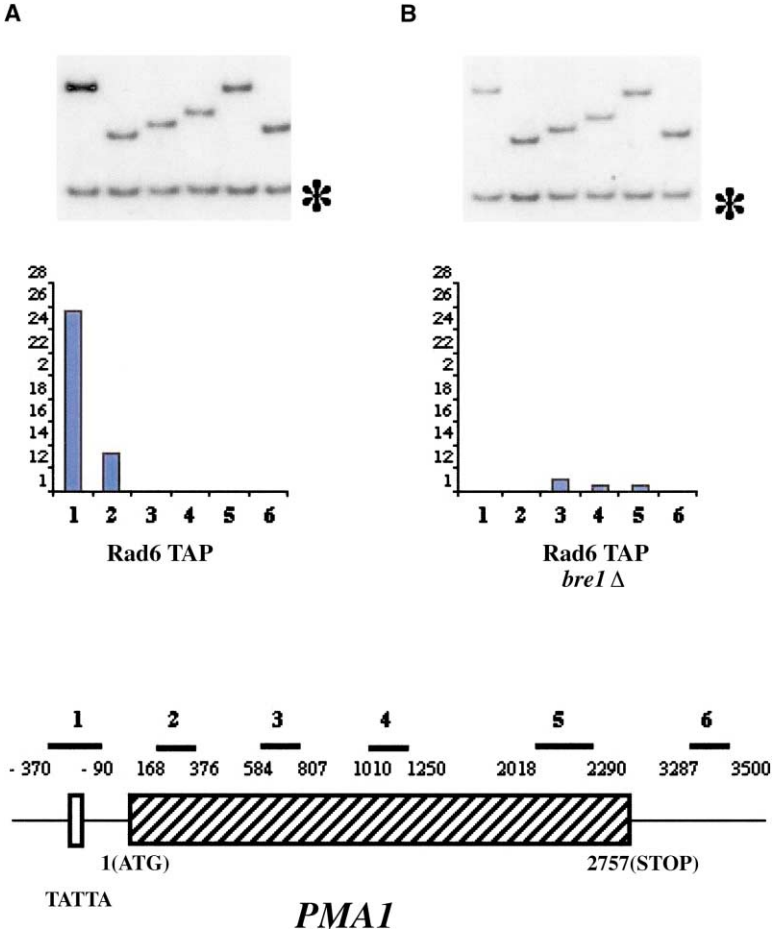

Figure 4. Bre1 Is Required for the Recruitment of Rad6 to a Promoter

Chromatin immunoprecipitation assays with Rad6-TAP and Rad6-TAP *bre1*Δ were performed as described previously. To monitor the presence of either Rad6 along the *PMA1* gene, chromatin was immunoprecipitated with rabbit IgG-agarose from a strain containing either Rad6-TAP or Rad6-TAP *bre1*Δ. PCR amplifications were carried out using primer pairs recognizing promoter (1), coding (2, 3, 4, and 5) and 3′ untranslated (6) regions for *PMA1*. Primer pairs are as follows: $PMA1_{-370}$ and $PMA1_{-90}$ (1), $PMA1_{168}$ and $PMA1_{376}$ (2), $PMA1_{584}$ and $PMA1_{807}$ (3), $PMA1_{1010}$ and $PMA1_{1250}$ (4), $PMA1_{2018}$ and $PMA1_{2290}$ (5), and $PMA1_{3287}$ and $PMA1_{3500}$ (6). Each PCR contained a second primer pair that amplified a region of chromosome V devoid of ORFs (*), thus providing an internal control for background. The ratio of the experimental to the control signal for the precipitated DNA was divided by the ratio of the experimental to the control signal for the input DNA.

nitrocellulose membrane, and probed with anti-methylhistone antisera at 1:1000 dilution, followed by detection of the bound antibody with horseradish peroxidase-conjugated to anti-rabbit IgG secondary antibodies (1:10,000 dilution). Anti-methyl Lys4 histone H3 was purchased from Upstate Biotechnology. Anti-ubiquitin antibody was purchased from Boston Biochemical.

Isolation of Yeast Extracts at Larger Quantities

To obtain yeast cell extracts in larger quantities, yeast cells were grown to mid-log phase in YPD medium, pelleted, washed with distilled water, pelleted, and resuspended in lysis buffer (20 mM Tris [pH 7.5], 50 mM KCl, 1 mM EDTA, 0.1% NP40, 1 mMDTT) and fresh protease and phosphatase inhibitors (1 μg/ml aprotinin, leupeptin, and pepstatin A; 1 mM PMSF; 1 μM microcystin-LR; 2 mM p-chloromercuriphenylsulfonic acid). Cells were then disrupted by vortexing with glass beads (0.5 mm; Biospec Products) for 15 min at 4°C. The bottoms of the microcentrifuge tubes were punctured, and cell extracts were recovered into a larger tube by brief centrifugation in a microfuge. The lysate was clarified by centrifugation at 20,000 g for 30 min, subjected to SDS-PAGE, transferred to nitrocellulose membrane, and probed with either anti-K4 methylhistone H3, anti-Flag, anti-ubiquitin, or anti-K79 methylhistone H3 antisera at about 1:1000 dilution, followed by detection of the bound antibody with horseradish peroxidase-conjugated to anti-rabbit IgG secondary antibodies (1:10,000 dilution).

Biochemical Purification of Rad6 and Bre1

Purification of the Bre1 and Rad6 containing complexes was carried out at 4°C. All purification steps were performed several times to assure the identification of correct polypeptides. Fractions were tested for Rad6 by Western analysis with anti-Rad6 polyclonal antibodies. For the affinity purification of complexes, both Bre1 and Rad6 were tagged by chromosomal integration via the C-terminal domain following a previously published method (Miller et al., 2001). Tagged complexes were purified essentially as described on IgG, and calmodulin columns from extracts were obtained from 5 liter yeast cultures grown in YPD medium to an OD_{600} of 1.0–1.5 as before (Miller et al., 2001). The protein bands were reduced, alkylated, and subjected to in-gel tryptic digestion, and the peptides were then purified and identified by MALDI-TOF spectrometry using a PerSeptive DE STR (Miller et al., 2001). Selected mass values from the MALDI-TOF experiments were taken to search the protein nonredundant database (NR; NCBI, Bethesda, MD) using the PeptideSearch algorithm. MS/MS spectra were inspected for y″ ion series to compare with the computer-generated fragment ion series of the predicted tryptic peptides.

Size Exclusion Chromatography on Superose 6PC

The conductivity of TAP-purified complexes was adjusted to a conductivity equivalent to that of 400 mM KCl in buffer A by dropwise addition of buffer C containing 1 M KCl. Samples were centrifuged at 14,000× g for 30 min and then applied to a Superose 6PC column (Pharmacia) equilibrated in buffer A containing 400 mM KCl. The column was eluted at 0.1 ml/min, and 100 ml fractions were collected. Fractions were subjected to SDS-PAGE and developed by silver staining.

In Vivo Analysis of Histone Ubiquitination

Strains expressing FLAG-tagged H2B as the only source of histone H2B were obtained from Dr. Struhl's laboratory (Ng et al., 2002a, 2002b). Both *rad6* and *bre1* deletions were made in this strain following published methods. Highly purified acid extracted histones from either wild-type strain or strains deficient for *rad6* or *bre1* were subjected to 16% SDS-PAGE electrophoresis and transferred to

nitrocellulose membrane, and probed with either anti-Flag or ubiquitin-specific antibodies followed by detection of the bound antibody with Horseradish peroxidase-conjugated to secondary antibodies (1:10,000 dilution).

Chromatin Immunoprecipitation

ChIP assays, in which proteins were crosslinked to DNA in vivo using formaldehyde, were employed to analyze the in vivo distribution of Rad6 and Bre1 along various regions of the PMA1 gene as described before (Krogan et al., 2002b). Following isolation and shearing of chromatin, Rad6-TAP was immunoprecipitated with IgG agarose (directed against protein A on the TAP tag). After reversal of the crosslinks, PCR analyses were performed on the coprecipitated DNA. Primer pairs directed against promoter regions, coding regions, and 3′ untranslated regions of the *PMA1* gene were used.

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