THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 14, pp. 9619–9633, April 5, 2013

Functional Analysis of Bre1p, an E3 Ligase for Histone H2B Ubiquitylation, in Regulation of RNA Polymerase II Association with Active Genes and Transcription *in Vivo*[*]

Received for publication, January 3, 2013, and in revised form, February 13, 2013 Published, JBC Papers in Press, February 15, 2013, DOI 10.1074/jbc.M113.450403

Rwik Sen, Shweta Lahudkar, Geetha Durairaj, and Sukesh R. Bhaumik[1]

From the Department of Biochemistry and Molecular Biology, Southern Illinois University, School of Medicine, Carbondale, Illinois 62901

Background: Bre1p is required for H2B ubiquitylation that promotes RNA polymerase II association with active genes, and hence transcription.
Results: The RING domain of Bre1p facilitates, but a non-RING domain represses, transcription and RNA polymerase II association with active genes.
Conclusion: Bre1p has a dominant-negative role in addition to its well known stimulatory function in transcription.
Significance: This study unravels a hidden role of Bre1p in transcriptional regulation.

H2B ubiquitylation is carried out by Bre1p, an E3 ligase, along with an E2 conjugase, Rad6p. H2B ubiquitylation has been previously implicated in promoting the association of RNA polymerase II with the coding sequence of the active *GAL1* gene, and hence transcriptional elongation. Intriguingly, we find here that the association of RNA polymerase II with the active *GAL1* coding sequence is not decreased in Δ*bre1*, although it is required for H2B ubiquitylation. In contrast, the loss of Rad6p significantly impairs the association of RNA polymerase II with *GAL1*. Likewise, the point mutation of lysine 123 (ubiquitylation site) to arginine of H2B (H2B-K123R) also lowers the association of RNA polymerase II with *GAL1*, consistent with the role of H2B ubiquitylation in promoting RNA polymerase II association. Surprisingly, unlike the Δ*rad6* and H2B-K123R strains, complete deletion of *BRE1* does not impair the association of RNA polymerase II with *GAL1*. However, deletion of the RING domain of Bre1p (that is essential for H2B ubiquitylation) impairs RNA polymerase II association with *GAL1*. These results imply that a non-RING domain of Bre1p counteracts the stimulatory role of the RING domain in regulating the association of RNA polymerase II with *GAL1*, and hence RNA polymerase II occupancy is not impaired in Δ*bre1*. Consistently, *GAL1* transcription is impaired in the absence of the RING domain of Bre1p, but not in Δ*bre1*. Similar results are also obtained at other genes. Collectively, our results implicate both the stimulatory and repressive roles of Bre1p in regulation of RNA polymerase II association with active genes (and hence transcription) *in vivo*.

Covalent modifications of histones are strongly associated with transcription (1–7). One such modification is the monoubiquitylation of histone H2B at lysine 123 (Lys-123) in yeast (Lys-120 in mammals). Histone H2B ubiquitylation has been implicated in transcriptional elongation (8–13), and thus, the association of RNA polymerase II with the coding sequence of the active gene is significantly decreased in the absence of histone H2B ubiquitylation (8–12). A complex of three proteins, Rad6p (E2 ubiquitin conjugase), Bre1p (E3 ubiquitin ligase) and Lge1p, is essential for histone H2B Lys-123 ubiquitylation in *Saccharomyces cerevisiae* (2, 4, 5, 13–19). This complex shows a very specific subset of synthetically fitness or lethal defect interactions with the SWR1 chromatin remodeling complex (9) and Sin3p/Rpd3p histone deacetylase (18). Furthermore, synthetically fitness or lethal defect interaction profiles and DNA damage sensitivity of the Δ*bre1* and Δ*lge1* mutants are almost identical (18).

Hypersensitivity of yeast deletion mutants to a drug, brefeldin A (which inhibits growth and secretion in eukaryotes by blocking transport between endoplasmic reticulum and golgi complex), has led to the identification of Bre1p (20). Later on, a null mutation in *BRE1* was also identified in a screen for deletion mutants, which are synthetically lethal with Δ*htz1* (*HTZ1* encodes H2A.Z in *S. cerevisiae*) (15). Wood *et al.* (16) have also identified Bre1p in a screen for histone H3 K4 methylation. These two later studies (15, 16) identified *BRE1* as an E3 ligase for histone H2B ubiquitylation. Furthermore, Bre1p has been characterized to direct Rad6p for histone H2B ubiquitylation at Lys-123 to promote transcriptional elongation (15, 16, 19). Without Bre1p, Rad6p ubiquitylates all histones nonspecifically (19, 21–23). Although histone H2B ubiquitylation by Rad6p-Bre1p has been implicated to promote transcriptional elongation (8–13), it is also involved in activating the Rad53 kinase and cell cycle arrest (DNA damage checkpoint response), gene silencing, replication stress, and apoptosis (24–32).

Yeast Bre1p forms a homomeric complex through multiple intermolecular interactions (19). It contains a C3HC4 zinc finger RING (really interesting new gene) domain near its C terminus. The RING domain is an E3 signature motif and is essen-

[*] This work was supported, in whole or in part, by National Institutes of Health Grant 1R15GM088798-01 (to S. R. B.), Grant-in-aid 10GRNT4300059 from the American Heart Association (Greater Midwest Affiliate), a Mallinckrodt Foundation grant, and Excellence in Academic Medicine awards of the Southern Illinois University School of Medicine.
[1] To whom correspondence should be addressed. Tel.: 618-453-6479; Fax: 618-453-6440; E-mail: sbhaumik@siumed.edu.

tial for histone H2B ubiquitylation (19). In addition to the roles of Rad6p and Bre1p in histone H2B ubiquitylation, polymerase II-associated factor 1 (Paf1)[2] complex (Paf1C) is also required for efficient histone H2B ubiquitylation (9, 33, 34). Paf1C that associates with elongating RNA polymerase II promotes the recruitment of Rad6p for efficient histone H2B ubiquitylation (9, 19). Furthermore, a recent study has demonstrated a direct interaction of Paf1C with Bre1p (19). Thus, RNA polymerase II-associated Paf1C plays an important role in promoting histone H2B ubiquitylation. Indeed, Shilatifard and colleagues (34) have demonstrated that Paf1C enhances histone H2B ubiquitylation activity of Rad6p-Bre1p.

As mentioned above, previous studies (8–13) have implicated histone H2B ubiquitylation in transcriptional elongation. Consistently, we have demonstrated that the absence of histone H2B ubiquitylation in the histone H2B-K123R point mutant strain significantly impaired the association of RNA polymerase II with the coding sequence of the *GAL1* gene following transcriptional induction (8). Therefore, it is expected that deletion of *BRE1* or *RAD6* would also lower the association of RNA polymerase II with *GAL1*, similar to the results in the histone H2B-K123R point mutant strain. Indeed, we find that the loss of Rad6p significantly impairs the association of RNA polymerase II with the *GAL1* coding sequence following transcriptional induction. Surprisingly, the deletion of *BRE1* does not decrease the association of RNA polymerase II with *GAL1*, even though Bre1p is essential for histone H2B ubiquitylation. However, deletion of the RING domain of Bre1p significantly impairs the association of RNA polymerase II with *GAL1*, consistent with the role of histone H2B ubiquitylation in association of RNA polymerase II and hence transcription. Thus, a non-RING domain of Bre1p appears to suppress the association of RNA polymerase II and counteracts the stimulatory role of the RING domain in RNA polymerase II association. Hence, the association of RNA polymerase II with *GAL1* is not impaired in the null mutation of *BRE1*. We also find similar results at several other genes such as *GAL10*, *ADH1*, and *RPS5*. Collectively, our results support both the stimulatory role (as expected based on previous studies; Refs. 8–13) as well as novel repressive function of Bre1p in regulation of RNA polymerase II association (and hence transcription) via its RING and non-RING domains. Thus, this study unravels a hidden function of Bre1p in gene regulation.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids pFA6a-13Myc-KanMX6 and pFA6a-3HA-His3MX6 (35) were used for genomic tagging of the proteins of interest by Myc and HA epitopes, respectively. The plasmid pRS406 (36) was used in the PCR-based gene disruption.

Strains—Yeast strain (*S. cerevisiae*) harboring point mutation in histone H2B at Lys-123 (YKH046) and its isogenic wild-type equivalent (YKH045) were obtained from the Osley laboratory (Mary Ann Osley, University of New Mexico Health Science Center) (31). The yeast strain bearing FLAG-tagged histone H2B (YTT31) was obtained from the Osley laboratory (37). The strain YTT31 was derived from JKM179 (Flag*HTB1*::*LEU2* in JKM179) (38). The yeast strain harboring the null mutation of *RAD6* (STY2; Δ*rad6* in FM392) and the wild-type equivalent (STY1; FM392) were obtained from the Shilatifard laboratory (Ali Shilatifard, Stowers Institute for Medical Research; purchased from Research Genetics). The *PAF1* deletion mutant strain (DY7014) in the W303a background was obtained from the Stillman laboratory (David Stillman, University of Utah Health Sciences Center). Wild-type (BY4741) and Δ*bre1* strains were obtained from the Davie laboratory (Judith K. Davie, SIU School of Medicine; purchased from Open Biosystems). The strain, SLY1a, was generated by deleting the *BRE1* gene in the YTT31 strain using the pRS406 plasmid. Multiple Myc epitope tags were added to the original chromosomal loci of *DST1* and *PAF1* in SLY1a to generate RSY20 and RSY17 strains, respectively, using the pFA6a-13Myc-KanMX6 plasmid. Similarly, multiple Myc epitope tags were added to the original chromosomal loci of *DST1* and *PAF1* in YTT31 to generate RSY19 and RSY16 strains, respectively, using the pFA6a-13Myc-KanMX6 plasmid. The HA epitope tags were added genomically to different locations toward the C-terminal of Bre1p using the pFA6a-3HA-KanMX6 plasmid in YTT31 to generate RSY12 (Bre1p), RSY13 (Bre1p-Δ50), RSY14 (Bre1p-Δ200), and RSY15 (Bre1p-Δ500) strains as schematically mentioned in Fig. 5*A*. Multiple Myc epitope tags were added to the original chromosomal locus of *PAF1* in the RSY12 and RSY13 strains to generate RSY21 and RSY22 strains, respectively, using the pFA6a-13Myc-Trp1 plasmid. Likewise, multiple Myc epitope tags were added to the original chromosomal locus of *DST1* in RSY13 to generate RSY24 strains.

Growth Media—The yeast strains were grown in YPR (yeast extract-peptone plus 2% raffinose) up to an A_{600} (optical density at 600 nm) of 0.9 at 30 °C, and then transferred to YPG (yeast extract, peptone plus 2% galactose) for 60 min to induce *GAL1* prior to formaldehyde-based *in vivo* cross-linking or harvesting for mRNA analysis. The *GAL10* gene was similarly induced. For studies at *RPS5* and *ADH1*, yeast cells were grown in YPD (yeast extract, peptone plus 2% dextrose) up to an A_{600} of 1.0 at 30 °C prior to cross-linking or harvesting for mRNA analysis.

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was performed as described previously (39–43). Briefly, yeast cells were treated with 1% formaldehyde, collected, and resuspended in lysis buffer. Following sonication, cell lysate (400 μl of lysate from 50 ml of yeast culture) was precleared by centrifugation, and then 100 μl of lysate was used for each immunoprecipitation. Immunoprecipitated protein-DNA complexes were treated with proteinase K, the cross-links were reversed, and DNA was purified. Immunoprecipitated DNA was dissolved in 20 μl of TE 8.0 (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and 1 μl of immunoprecipitated DNA was analyzed by PCR. PCR contained [α-^{32}P]dATP (2.5 μCi for 25 μl of reaction), and the PCR products were detected by autoradiography after separation on a 6% polyacrylamide gel. As a control, "input" DNA was isolated from 5 μl of lysate without going through the immunoprecipitation step, and dissolved in 100 μl of TE 8.0. To compare the PCR signal arising from the immu-

[2] The abbreviations used are: Paf1, polymerase II-associated factor 1; MMS, methyl methanosulfonate; HU, hydroxy urea.

noprecipitated DNA with the input DNA, 1 μl of input DNA was used in the PCR analysis.

For analysis of the recruitment of Dst1p and Paf1p, the above ChIP protocol was modified as described previously (43, 44). Briefly, a total of 800 μl of lysate was prepared from 100 ml of yeast culture. Following sonication, 400 μl of lysate was used for each immunoprecipitation (using 10 μl of anti-Myc antibody and 100 μl of protein A/G plus-agarose beads from Santa Cruz Biotechnology, Inc.), and the immunoprecipitated DNA sample was dissolved in 10 μl of TE 8.0 of which 1 μl was used for the PCR analysis. In parallel, the PCR analysis for input DNA was performed using 1 μl of DNA that was prepared by dissolving purified DNA from 5 μl of lysate in 100 μl of TE 8.0. The primer pairs used for PCR analysis were as follows: *GAL1* (Core), 5′-ATAGGATGATAATGCGATTAGTTTTTTAGCCTT-3′ and 5′-GAAAATGTTGAAAGTATTAGTTAAAGTGGTTATGCA-3′; *GAL1* (ORF1), 5′-CAGTGGATTGTCTTCTTCGGCCGC-3′ and 5′-GGCAGCCTGATCCATACCGCCATT-3′; *GAL1* (ORF2), 5′-CAGAGGGCTAAGCATGTGTATTCT-3′ and 5′-GTCAATCTCTGGACAAGAACATTC-3′; *GAL10* (ORF1), 5′-CTACGAGATTCCCAAATATGATTCC-3′ and 5′-TAACGCAAGATAGCAAACTTCCAAC-3′; *GAL10* (ORF2), 5′-TTAATGCGAATCATAGTAGTATCGG-3′ and 5′-TTACCAATAGATCACCTGGAAATTC-3′; *ADH1* (ORF), 5′-CTGGTTACACCCACGACGGTTCTT-3′ and 5′-GCAGACTTCAAAGCCTTGTAGACG-3′; *RPS5* (ORF), 5′-AGGCTCAATGTCCAATCATTGAAAG-3′ and 5′-CAACAACTTGGATTGGGTTTTGGTC-3′. Autoradiograms were scanned and quantitated by NIH Image 1.62. Immunoprecipitated DNAs were quantitated as the ratio of immunoprecipitate to input in the autoradiogram (ORF, open reading frame; and core, core promoter). ORF1 and ORF2 represent two different locations in the ORF.

The ChIP experiments were carried out three times. These experiments are biologically independent. The average ChIP signal of these biologically independent experiments is reported with standard deviation (S.D.; Microsoft Excel 2003). The Student's *t* test of Microsoft Excel 2003 (with tail = 2 and types = 3) was used to determine the *p* values for statistical significance of the change in the ChIP signals. The changes were considered to be statistically significant at $p < 0.05$.

Total RNA Preparation—The total RNA was prepared from yeast cell culture as described by Peterson *et al.* (45). Briefly, 10 ml of yeast culture was harvested, and then suspended in 100 μl of RNA preparation buffer (500 mM NaCl, 200 mM Tris-HCl, 100 mM Na_2EDTA, and 1% SDS) along with 100 μl of phenol/chloroform/isoamyl alcohol and a 100-μl volume-equivalent of glass beads (acid washed; Sigma). Subsequently, yeast cell suspension was vortexed with a maximum speed (10 in VWR mini-vortexer; catalog number 58816-121) 5 times (30 s each). Cell suspension was put in ice for 30 s between pulses. After vortexing, 150 μl of RNA preparation buffer and 150 μl of phenol/chloroform/isoamyl alcohol were added to yeast cell suspension followed by vortexing for 15 s with a maximum speed on VWR mini-vortexer. The aqueous phase was collected following a 5-min centrifugation at a maximum speed in a microcentrifuge. The total RNA was isolated from aqueous phase by ethanol precipitation.

Reverse Transcriptase-PCR (RT-PCR) Analysis—RT-PCR analysis was performed according to the standard protocols (46). Briefly, total RNA was prepared from 10 ml of yeast culture. Ten micrograms of total RNA was used in the reverse transcription assay for both wild-type and mutant strains. RNA was treated with RNase-free DNase (M610A, Promega) and then reverse-transcribed into cDNA using oligo(dT) as described in the protocol supplied by Promega (A3800, Promega). PCR was performed using synthesized first strand as template and the primer pairs targeted to *GAL1, GAL10, ADH1*, and *RPS5* ORFs. RT-PCR products were separated by 2.2% agarose gel electrophoresis and visualized by ethidium bromide staining. The primer pairs used in the PCR analysis were as follows: *GAL1,* 5′-CAGAGGGCTAAGCATGTGTATTCT-3′, 5′-GTCAATCTCTGGACAAGAACATTC-3′; *GAL10,* 5′-TTAATGCGAATCATAGTAGTATCGG-3′, 5′-TTACCAATAGATCACCTGGAAATTC-3′; *ADH1,* 5′-CGGTAACAGAGCTGACACCAGAGA-3′, 5′-ACGTATCTACCAACGATTTGACCC-3′; *RPS5,* 5′-AGGCTCAATGTCCAATCATTGAAAG-3′, 5′-CAACAACTTGGATTGGGTTTTGGTC-3′. The RT-PCR experiments were carried out three times. These experiments are biologically independent. The average signal of these biologically independent experiments is reported with S.D. (Microsoft Excel 2003). The Student's *t* test (with tail = 2 and types = 3) was used to determine *p* values for statistical significance of the change in the RT-PCR signals. The changes were considered to be statistically significant at $p < 0.05$.

Whole Cell Extract Preparation and Western Blot Analysis—For analysis of the global levels of Bre1p mutants, yeast strains expressing the HA epitope-tagged proteins were grown in 25 ml of YPD up to an A_{600} of 1.0, and then harvested cells were lysed in 100 μl of lysis buffer to prepare the whole cell extract following the protocol as described for the ChIP assay (39–44). The whole cell extract (12.5 μl) was run on a SDS-polyacrylamide gel, and then analyzed by Western blot assay. The anti-HA (Santa Cruz Biotechnology, Inc.) antibody was used as a primary antibody in Western blot analysis. The level of Actin was monitored as a loading control using an anti-actin antibody (A2066; Sigma). For analysis of global levels of Paf1p in the wild-type and Bre1p null mutant strains, yeast strains expressing Myc epitope-tagged Paf1p were grown in 10 ml of YPR up to an A_{600} of 0.9, and then switched to YPG for 60 min at 30 °C prior to harvesting. The harvested cells were lysed in 100 μl of lysis buffer to prepare the whole cell extract. 10 μl of whole cell extract was used for Western blot analysis using an anti-Myc (9E10; Santa Cruz Biotechnology, Inc.) antibody. For analysis of histone H2B ubiquitylation, yeast cells were grown in YPD at 30 °C up to an A_{600} of 1.0. 5 ml of yeast culture was harvested, washed, and boiled with 70 μl of 1× SDS-polyacrylamide gel loading buffer for electrophoresis, as described previously (15). Western blot analysis was performed using an anti-FLAG antibody against FLAG-tagged histone H2B.

Growth Analysis following Genotoxic Attacks—The growth of the Δ*rad6*, Δ*bre1*, and wild-type cells was analyzed on plates containing solid YPD plus 0.026% MMS (methyl methanosulfonate; 129925–5G, Sigma) or 100 mM HU (hydroxy urea; H8627-5G; Sigma). Yeast cells were inoculated in YPD, and grown up to an A_{600} of 1.0 without dilution. Yeast cells were

then spotted on solid growth media following serial dilutions. Yeast cells were grown at 30 °C, and photographed after 2 or 3 days.

RESULTS

Association of RNA Polymerase II with GAL1 Is Not Impaired in the Δbre1 Strain, whereas Histone H2B K123 Ubiquitylation or Rad6p Facilitates GAL1 Association of RNA Polymerase II—Previous studies have implicated histone H2B ubiquitylation in promoting RNA polymerase II to passage through nucleosomes at the active coding sequence, and hence transcriptional elongation (8, 10, 11). Thus, the association (or occupancy) of RNA polymerase II with the coding sequence of the active gene would be impaired in the absence of the enzymes (Rad6p and Bre1p) involved in this covalent modification. Indeed, we find that deletion of *RAD6* significantly decreased the association of RNA polymerase II with the coding sequence of the *GAL1* gene following transcriptional induction in galactose-containing growth medium (Fig. 1, *A* and *B*), consistent with the role of histone H2B ubiquitylation in transcriptional elongation (8–12). Similar to the Δ*rad6* strain, mutation of Lys-123 of histone H2B to arginine (H2B-K123R) also decreased the association of RNA polymerase II with *GAL1* (Fig. 1*C*), consistent with previous studies (8, 10, 12). These results demonstrate that histone H2B ubiquitylation promotes the association of RNA polymerase II with *GAL1*. Consistently, histone H2B ubiquitylation has been shown to promote *GAL1* transcription (8–10). Furthermore, recent genome-wide studies have also implicated histone H2B ubiquitylation in promoting transcription (11). Because Bre1p is essential for histone H2B ubiquitylation (15, 16, 19), the association of RNA polymerase II with *GAL1* would also be impaired in the Δ*bre1* strain, similar to the results obtained in the Δ*rad6* and H2B-K123R mutant strains (Fig. 1, *B* and *C*). Surprisingly, we did not observe the decrease in RNA polymerase II association with the *GAL1* coding sequence following transcriptional induction in the Δ*bre1* strain (Fig. 1*D*), even though it is essential for histone H2B ubiquitylation (15, 16, 19). Thus, our results (Figs. 1, *B–D*) demonstrate that the complete deletion of *BRE1* does not decrease the association of RNA polymerase II with *GAL1*, whereas the absence of histone H2B ubiquitylation or E2 conjugase Rad6p significantly impairs RNA polymerase II association.

GAL1 Transcription Is Impaired in Δrad6 and H2B K123R Mutant Strains, but Not in the Δbre1 Strain—Because RNA polymerase II association is correlated with transcription, *GAL1* transcription would be impaired in Δ*rad6* and H2B K123R mutant strains. To test this, we analyzed *GAL1* mRNA levels in Δ*rad6* and H2B K123R mutant strains and their isogenic wild-type equivalents. Our RT-PCR analysis revealed that *GAL1* transcription was significantly impaired in Δ*rad6* and H2B K123R mutant strains in comparison to wild-type equivalents (Fig. 1*E*). However, transcription of *GAL1* was not impaired in the Δ*bre1* strain (Fig. 1*E*). These transcription results are nicely correlated with RNA polymerase II association with *GAL1* in Δ*bre1*, Δ*rad6*, and H2B K123R mutant strains.

The Δ*bre1* strain used in our study might have contained some hidden mutation, which has reversed the defect in the RNA polymerase II association phenotype with *GAL1*. To address this issue, we also used a commercial Δ*bre1* strain and its isogenic wild-type equivalent (from Open Biosystems) for analysis of RNA polymerase II association with *GAL1* and transcription. Using this commercial Δ*bre1* strain, we again found that association of RNA polymerase II with *GAL1* was not impaired in the absence of Bre1p (Fig. 1*F*). Consistently, transcription of *GAL1* was not altered in the commercial Δ*bre1* strain (Fig. 1*G*). Thus, the Δ*bre1* strain used in our study does not appear to contain a hidden mutation. Similar to our results, a recent study in *S. cerevisiae* also demonstrated that association of RNA polymerase II with *GAL10* and *PMA1* was not decreased in the Δ*bre1* strain (14).

As presented above, unlike Δ*rad6* and H2B K123R mutants, we do not observe an impairment of RNA polymerase II association with *GAL1* (and hence transcription) in the Δ*bre1* strain in comparison to the wild-type equivalent. These observations raised the possibility that there may be another redundant histone H2B ubiquitin ligase. However, we rule out this possibility as previous studies (15, 16, 19) have demonstrated a dramatic impairment of histone H2B ubiquitylation in the Δ*bre1* strain. Consistently, we also do not observe histone H2B ubiquitylation in the absence of Bre1p or its RING domain (Fig. 1*H*). Furthermore, similar to the Δ*rad6* strain, the Δ*bre1* strain shows sensitivity to genotoxic agents such as MMS and HU (Fig. 1*I*), consistent with previous studies (16, 25, 47).

The RING Domain of Bre1p Promotes RNA Polymerase II Association with GAL1 and hence Transcription—Why does the null mutation of *BRE1* not show an impaired association of RNA polymerase II with *GAL1* (and hence transcription), similar to the results in the Δ*rad6* and H2B-K123R mutant strains? It is likely that the RING domain of Bre1p (that is essential for histone H2B ubiquitylation; Ref. 19) promotes RNA polymerase II association via histone H2B ubiquitylation, like E2 conjugase Rad6p, and a non-RING domain is involved in repression of the association of RNA polymerase II. Hence, the decrease in association of RNA polymerase II in the Δ*bre1* strain was not observed due to counteracting of the stimulatory role of the RING domain by the repressive role of a non-RING domain of Bre1p. To test this possibility, we deleted the RING domain of Bre1p (Fig. 2*A*) following HA-epitope tagging at the genomic locus of *BRE1*, and then analyzed the effect of such a deletion on RNA polymerase II association with *GAL1*. Interestingly, we find that the association of RNA polymerase II with the *GAL1* coding sequence was significantly decreased in the absence of the RING domain of Bre1p (Fig. 2*B*). The RING domain is essential for histone H2B ubiquitylation (19) (Fig. 1*H*). Thus, histone H2B ubiquitylation activity of Bre1p is essential to promote the association of RNA polymerase II with *GAL1*, similar to the RNA polymerase II association phenotype in Δ*rad6* and histone H2B-K123R mutant strains (Fig. 1, *B* and *C*). Consistently, transcription of *GAL1* was significantly impaired in the absence of the RING domain of Bre1p (Fig. 2*C*). Furthermore, deletion of the RING domain does not alter the interaction of Bre1p with Rad6p (19). However, Bre1p without the RING domain is relatively less stable (Fig. 2*D*), but does not generate the phenotypes of the Δ*bre1* strain. Thus, even at the lower level, the Bre1p mutant without the RING domain exhibits a repressive function. Consistently, it has been demonstrated

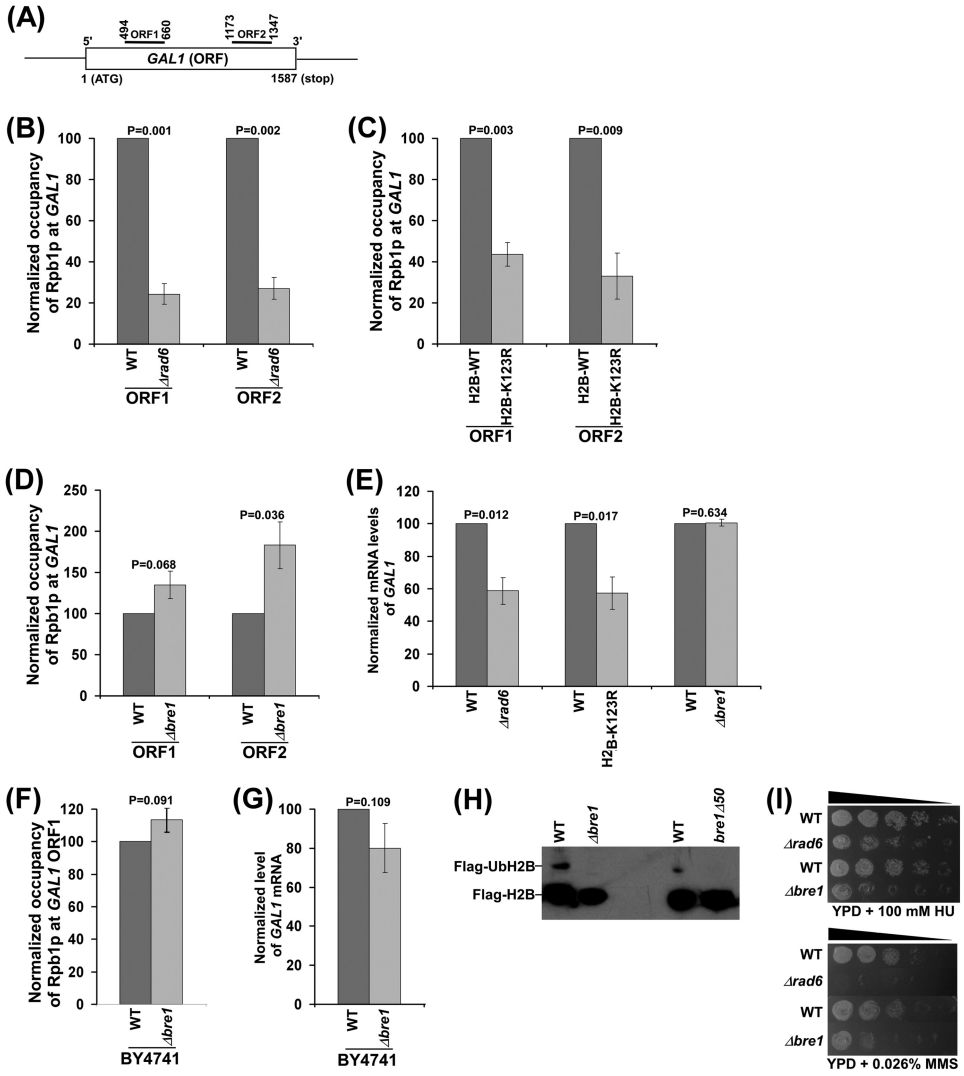

FIGURE 1. **Analysis of the association of RNA polymerase II with *GAL1*.** *A,* a schematic diagram showing the locations of the primer pairs (ORF1 and ORF2) at the *GAL1* coding sequence for the ChIP analysis. The *numbers* are presented with respect to the position of the first nucleotide of the initiation codon (+1). *B,* Rad6p promotes the association of RNA polymerase II with *GAL1*. Yeast strains (STY1 and STY2) were grown in YPR at 30 °C up to an A_{600} of 0.9, and then switched to YPG for 60 min prior to formaldehyde-based *in vivo* cross-linking. The ChIP assay was performed as described under "Experimental Procedures." Immunoprecipitation was performed using 8WG16 antibody (Covance, Inc.) against the carboxyl-terminal domain of the largest subunit (Rpb1p) of RNA polymerase II. Immunoprecipitated DNA was analyzed by PCR using the primer pairs located at two different locations of the *GAL1* coding sequence (ORF1 and ORF2). The ratio of the immunoprecipitate over the input in the autoradiogram (termed as a ChIP signal) was measured. The ChIP signal of the wild-type strain was set to 100, and the ChIP signal of the mutant strain was normalized with respect to 100 (represented as normalized or relative occupancy). *Error bars* denote S.D. from three sets of biologically independent experiments. *p* values were calculated using the Student's *t* test. *C,* association of RNA polymerase II with *GAL1* is significantly decreased in the histone H2B-K123R point mutant strain. Yeast strains (YKH045 and YKH046) were grown, cross-linked, and immunoprecipitated as in *panel B*. *D,* association of RNA polymerase II with *GAL1* is not impaired in the absence of Bre1p. Yeast strains (YTT31 and SLY1a) were grown, cross-linked, and immunoprecipitated as in *panel B*. *E,* analysis of the *GAL1* transcription by RT-PCR. The wild-type and mutant strains were grown as in *panel B*. The transcript level of the wild-type strain was set to 100, and the mRNA level in the mutant strain was normalized with respect to 100. *F* and *G,* analysis of RNA polymerase II association with *GAL1* and transcription in the commercial Δ*bre1* and isogenic wild-type (BY4741) strains (from Open Biosystems). Both wild-type and mutant strains were grown as in *panel B*. *H*, Western blot analysis for histone H2B ubiquitylation in the presence and absence of Bre1p or its RING domain, using an anti-FLAG antibody against FLAG-tagged histone H2B. The *bre1Δ50* strain (RSY13) represents Bre1p without its RING domain. *UbH2B*, ubiquitylated histone H2B; and *Flag-H2B,* FLAG-tagged histone H2B. *I,* growth analysis of the Δ*rad6*, Δ*bre1*, and isogenic wild-type strains in solid YPD containing 100 mM HU or 0.026% MMS.

previously that the global level of a protein may not be directly correlated with its targeted function. For example, TATA-box binding protein is not recruited to the *GAL1* core promoter in the absence of Gal4p, whereas the global level of TATA-box binding protein is not altered in Δ*gal4* (39, 42). Similarly, Tra1p is not recruited to the *GAL1* upstream activating sequence in the absence of Spt20p, whereas its global level is not altered in Δ*spt20* (42). Deletion of RING and the adjacent coiled-coil domain does not impair the stability of the mutant (see below; Fig. 5*B*) and lowers the association of RNA polymerase II and transcription (see below; Fig. 5, *C* and *D*), similar to the Δ*rad6* and H2B K123R mutant strains. Together, these results support that like Rad6p, the RING finger with/without the adjacent coiled-coil domain of Bre1p (or its histone H2B ubiquitylation activity; Ref. 19) (Fig. 1*H*) is essential for promoting the association of RNA polymerase II with the active *GAL1* coding sequence. Interestingly, deletion of the whole *BRE1* reverses the defect in RNA polymerase II association caused by the removal

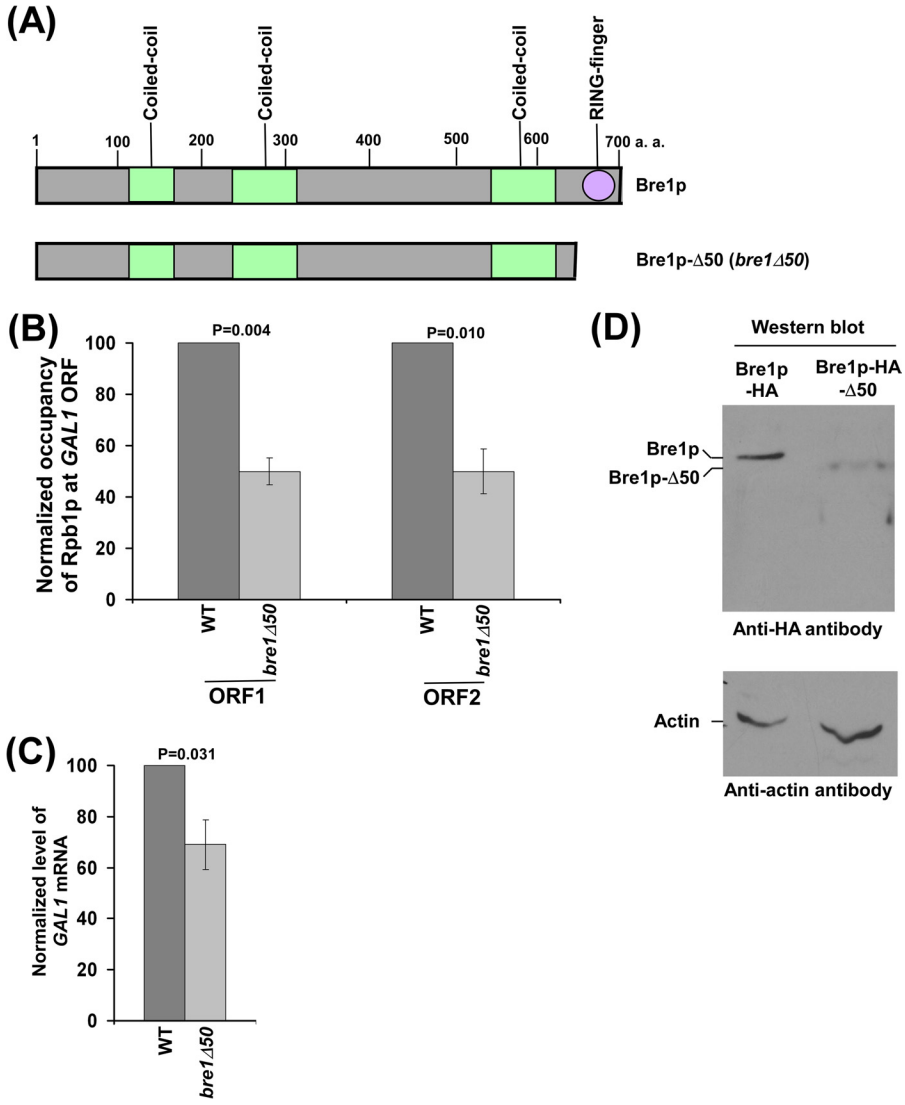

FIGURE 2. **The RING domain of Bre1p promotes the association of RNA polymerase II with *GAL1*.** *A,* schematic diagram of the Bre1p mutant without the RING domain. *B*, analysis of RNA polymerase II association with *GAL1* in the presence and absence of the RING domain of Bre1p. Yeast strains (RSY12 and RSY13) were grown, cross-linked, and immunoprecipitated as described in the legend to Fig. 1*B*. *C*, analysis of *GAL1* transcription by RT-PCR. Yeast cells were grown as described in the legend to Fig. 1*B*. *D,* Western blot analysis.

of the RING finger of Bre1p (Figs. 1*D* and 2*B*) and the adjacent coiled-coil domain (see below; Fig. 5*C*). These results support that a domain within the non-RING domain at the N-terminal of Bre1p is involved in repression of RNA polymerase II association. As a result of the two opposing effects of the RING and non-RING domains of Bre1p, we do not observe the decrease in RNA polymerase II association with *GAL1* (and hence transcription) in the null mutant of *BRE1*. However, the Δ*bre1* strain shows MMS and HU sensitivity, similar to the Δ*rad6* strain (Fig. 1*I*). MMS causes DNA damage, whereas HU generates replication stress. Thus, the repressive role of Bre1p appears to be restricted to transcription, but not DNA repair or replication. Likewise, a recent study in *S. cerevisiae* also showed that the association of RNA polymerase II with *GAL10* and *PMA1* was not decreased in the null mutant of *BRE1* in comparison to the wild-type equivalent (14). On the other hand, the association of RNA polymerase II with these genes was significantly impaired in the H2B-K123R point mutant strain (14). Therefore, similar to our data, the results of a recent study (14) also implicated the repressive role of Bre1p in association of RNA polymerase II with the active gene.

Bre1p or Its RING Domain Does Not Regulate the Recruitment of TFIIS to GAL1—How does Bre1p exert its repressive role in association of RNA polymerase II? A recent study (48) in mammalian cells demonstrated a set of genes whose expression was repressed by RNF20 (human paralog of yeast Bre1p). This study further demonstrates that such suppression is mediated via an impaired recruitment of TFIIS that promotes transcription (48). To test whether Bre1p exerts its repressive role in association of RNA polymerase II by inhibiting the recruitment of TFIIS in yeast, we analyzed the association of TFIIS to *GAL1* in the presence and absence of Bre1p. In this direction, we tagged TFIIS (*DST1*) by the Myc epitope in wild-type and Δ*bre1* mutant strains. Using these strains, we analyzed the recruitment of TFIIS to *GAL1* following transcriptional induction. We

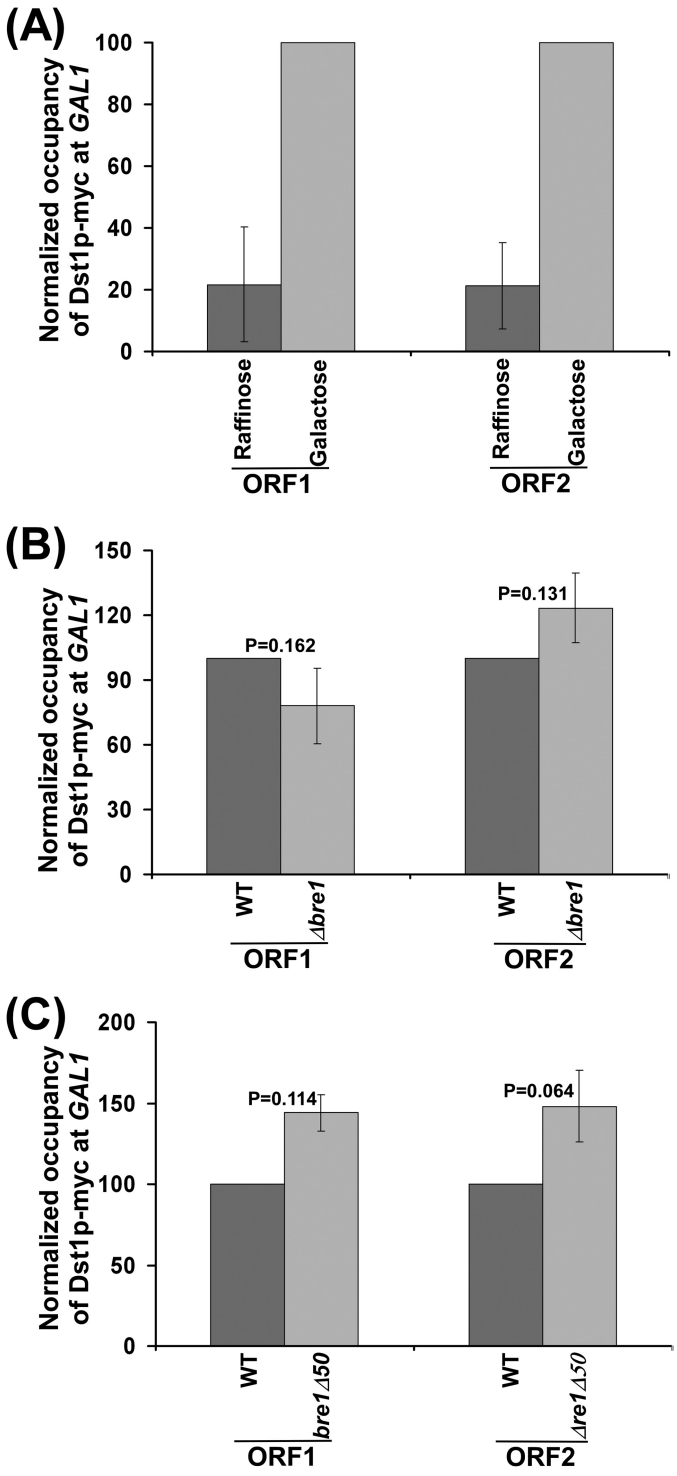

FIGURE 3. **Association of TFIIS (Dst1p) with *GAL1* is not altered in the absence of Bre1p or its RING domain.** *A*, analysis of the association of TFIIS with *GAL1* under inducible (galactose) and non-inducible (raffinose) conditions. Yeast strain expressing Myc-tagged Dst1p (RSY19) was grown at 30 °C in YPR up to an A_{600} of 0.9 prior to formaldehyde-based *in vivo* cross-linking. For *GAL1* induction, a yeast strain (RSY19) was grown at 30 °C in YPR up to A_{600} of 0.9, and then transferred to YPG for 60 min prior to formaldehyde-based *in vivo* cross-linking. The ChIP assay was performed following the modified protocol as described under "Experimental Procedures." Immunoprecipitation was carried out using an anti-Myc antibody (9E10; Santa Cruz Biotechnology, Inc.) against Myc-tagged Dst1p. The ChIP signals at ORF1 and ORF2 in galactose (or YPG) were set to 100. The ChIP signals at ORF1 and ORF2 in raffinose (or YPR) were normalized with respect to 100 (represented as normalized or relative occupancy). *Error bars* in YPR denote S.D. from three sets of biologically independent experiments. *B,* analysis of the association of TFIIS with *GAL1* in the presence and absence of Bre1p. Both the wild-type and Δ*bre1* strains expressing Myc-tagged Dst1p (RSY19 and RSY20) were grown and cross-linked as described in the legend to Fig. 1*B*. Immunoprecipitation was performed as in *panel A*. *C*, analysis of the association of TFIIS with *GAL1* in the presence and absence of the RING domain of Bre1p. Yeast cells were grown, cross-linked, and immunoprecipitated as in *panel B*.

find that recruitment of TFIIS to *GAL1* was not significantly altered in the absence of Bre1p (Fig. 3, *A* and *B*). Similarly, deletion of the RING domain of Bre1p did not impair the recruitment of TFIIS (Fig. 3*C*). Thus, Bre1p does not appear to function through TFIIS in yeast.

Bre1p Promotes the Recruitment of Paf1C to GAL1—Because the role of Bre1p in regulation of RNA polymerase II association is not mediated via TFIIS, it may be likely that the non-RING domain of Bre1p interacts with other factors that regulate the association of RNA polymerase II. The non-RING domain of Bre1p has been recently shown to interact with Rad6p (19), and such an interaction is essential for histone H2B ubiquitylation in conjunction with the RING domain (19, 34). Thus, deletion of *RAD6* or the RING domain of Bre1p significantly impaired the association of RNA polymerase II (Figs. 1*B* and 2*B*; see below, Fig. 5*C*), via the loss of histone H2B ubiquitylation (19). Recently, Kim and Roeder (19) demonstrated that Bre1p interacts with Paf1C. Paf1C associates with elongating RNA polymerase II, and promotes transcriptional elongation (33, 49–53). The Paf1C has also been previously implicated in histone H2B ubiquitylation (9, 33, 34). Therefore, there is a link between the general transcription machinery and histone H2B ubiquitylation. Interestingly, we find here that the recruitment of Paf1C (Paf1p) to *GAL1* was significantly decreased in the Δ*bre1* strain (Fig. 4, *A* and *B*). Such a decrease in the recruitment of Paf1C in the absence of Bre1p was not due to an altered stability of Paf1p in the absence of Bre1p as evident from Western blot analysis (Fig. 4*C*). Thus, our results support that Bre1p promotes the recruitment of Paf1C. It is important to note here that association of RNA polymerase II with *GAL1* in the Δ*bre1* strain was not decreased (Fig. 1*D*), but recruitment of RNA polymerase II-associated Paf1C was significantly impaired in the absence of Bre1p (Fig. 4*B*). Thus, the association of Paf1C with the active gene depends on Bre1p in addition to RNA polymerase II. This is further substantiated by the fact that Bre1p interacts with Paf1C (19). Therefore, our results demonstrate the function of Bre1p in targeting the recruitment of Paf1C in addition to the role of RNA polymerase II *in vivo*.

Paf1C Promotes RNA Polymerase II Association with GAL1, and hence Transcription—Our results demonstrate that Bre1p promotes the recruitment of Paf1C. Previous studies have demonstrated that Paf1C is required for recruitment of the E2 conjugase Rad6p (9, 19). Therefore, Paf1C has been implicated in histone H2B ubiquitylation (9, 33, 34). Hence, deletion of *PAF1* would decrease the association of RNA polymerase II via an impaired histone H2B ubiquitylation. To test this, we analyzed the association of RNA polymerase II with the *GAL1* coding sequence in the Δ*paf1* and wild-type strains. As expected, we find that association of RNA polymerase II with *GAL1* was significantly decreased in the Δ*paf1* strain (Fig. 4*D*). Consis-

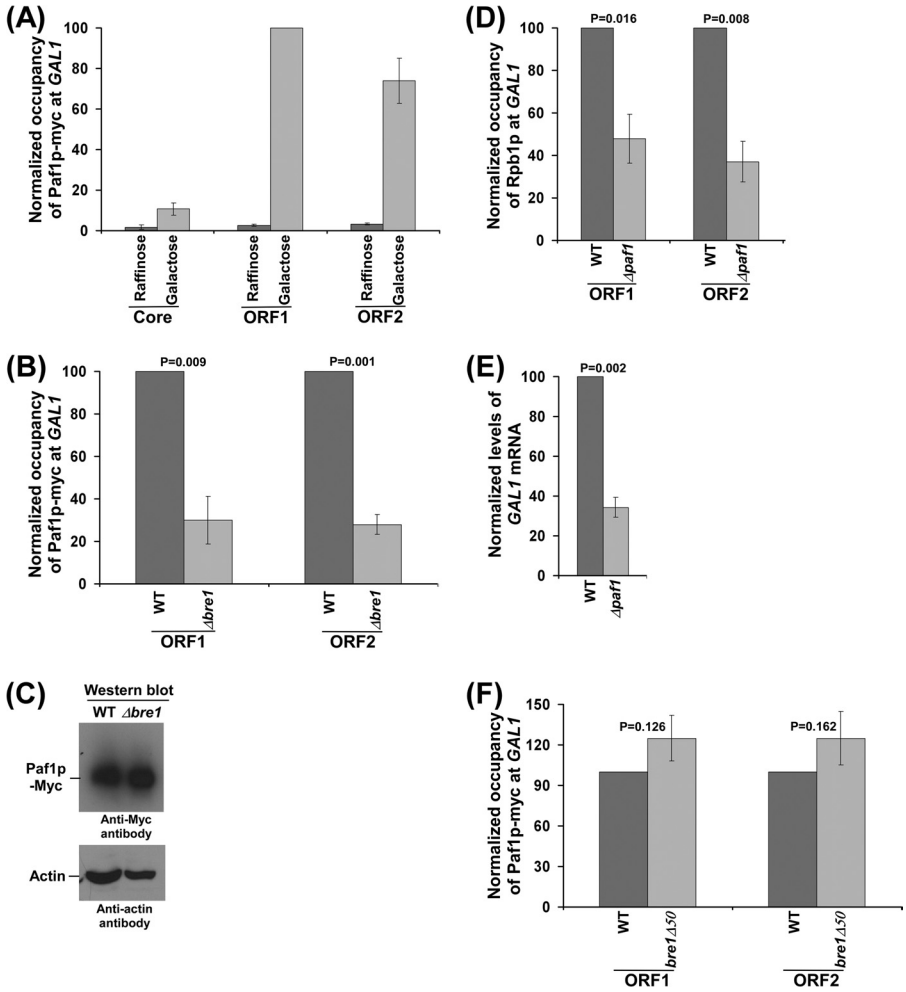

FIGURE 4. **Analysis of Paf1p recruitment to *GAL1* in the presence and absence of Bre1p.** *A*, analysis of Paf1p association with *GAL1* under inducible and non-inducible conditions. Wild-type yeast strain expressing Myc-tagged Paf1p (RSY16) was grown, cross-linked, and immunoprecipitated as described in the legend to Fig. 3*A*. The maximum ChIP signal was set to 100, and other ChIP signals were normalized with respect to 100 (represented as normalized or relative occupancy). *Error bars* denote S.D. from three sets of biologically independent experiments. *B,* association of Paf1p with *GAL1* is significantly decreased in the Δ*bre1* strain. Both the wild-type and Δ*bre1* strains expressing Myc-tagged Paf1p (RSY16 and RSY17) were grown, cross-linked, and immunoprecipitated as in Fig. 3*B*. *C*, Western blot analysis. Yeast cells were grown as described in the legend to Fig. 1*B*. *D,* Paf1p promotes the association of RNA polymerase II with *GAL1*. Both the wild-type and Δ*paf1* strains (W303a and DY7014) were grown, cross-linked, and immunoprecipitated as described in the legend to Fig. 1*B*. *E,* analysis of *GAL1* transcription in the Δ*paf1* strain by RT-PCR. Yeast cells were grown as described in the legend to Fig. 1*B*. *F,* the RING domain of Bre1p does not regulate the recruitment of Paf1C to *GAL1*. Both the wild-type and mutant strains expressing Myc-tagged Paf1p (RSY21 and RSY22) were grown and cross-linked as described in the legend to Fig. 1*B*. Immunoprecipitation was performed as in *panel A*.

tently, *GAL1* transcription was impaired in the Δ*paf1* strain (Fig. 4*E*).

The RING Domain of Bre1p Is Dispensable for Recruitment of Paf1C to GAL1—We observe that deletion of Bre1p significantly impairs the recruitment of Paf1C to *GAL1*. We next analyzed whether the RING domain of Bre1p plays a role in targeting Paf1C to *GAL1*. We find that recruitment of Paf1C was not altered in the absence of the RING domain of Bre1p (Fig. 4*F*). Thus, the non-RING domain of Bre1p facilitates the recruitment of Paf1C. Hence, the non-RING domain of Bre1p appears to interact with Paf1C and promotes RNA polymerase II association. Therefore, the non-RING domain of Bre1p has a stimulatory role in addition to its repressive role in controlling the association of RNA polymerase II.

Functional Analysis of Different Domains of Bre1p in Association of RNA Polymerase II with GAL1—To determine the region within the non-RING domain of Bre1p involved in repression of RNA polymerase II association, we generated two Bre1p constructs containing first 200 or 500 amino acids at the N-terminal (Fig. 5*A*) following HA epitope tagging at the genomic locus of *BRE1*. These Bre1p mutants with 200 or 500 amino acids at the N-terminal were found to be stable in the Western blot analysis (Fig. 5*B*). Using these mutants, we analyzed the association of RNA polymerase II with the *GAL1* coding sequence in conjunction with the RING-deficient Bre1p mutant and wild-type strains. We find that like the RING-deficient Bre1p mutant, Bre1p with 200 or 500 amino acids at the N-terminal also significantly reduced the association of RNA polymerase II with *GAL1* (Fig. 5*C*). Consistently, *GAL1* transcription was significantly impaired in these mutants (Fig. 5*D*). However, deletion of the whole *BRE1* reversed the defect of RNA polymerase II association with *GAL1* (and hence transcription), caused by the loss of the RING domain or deletion of 200/500 amino acids at the C-terminal of Bre1p (Figs. 1, *D* and *E*, and 5, *C* and *D*). These results support that the first ~200 amino acids at the N-terminal of Bre1p exhibits the repressive

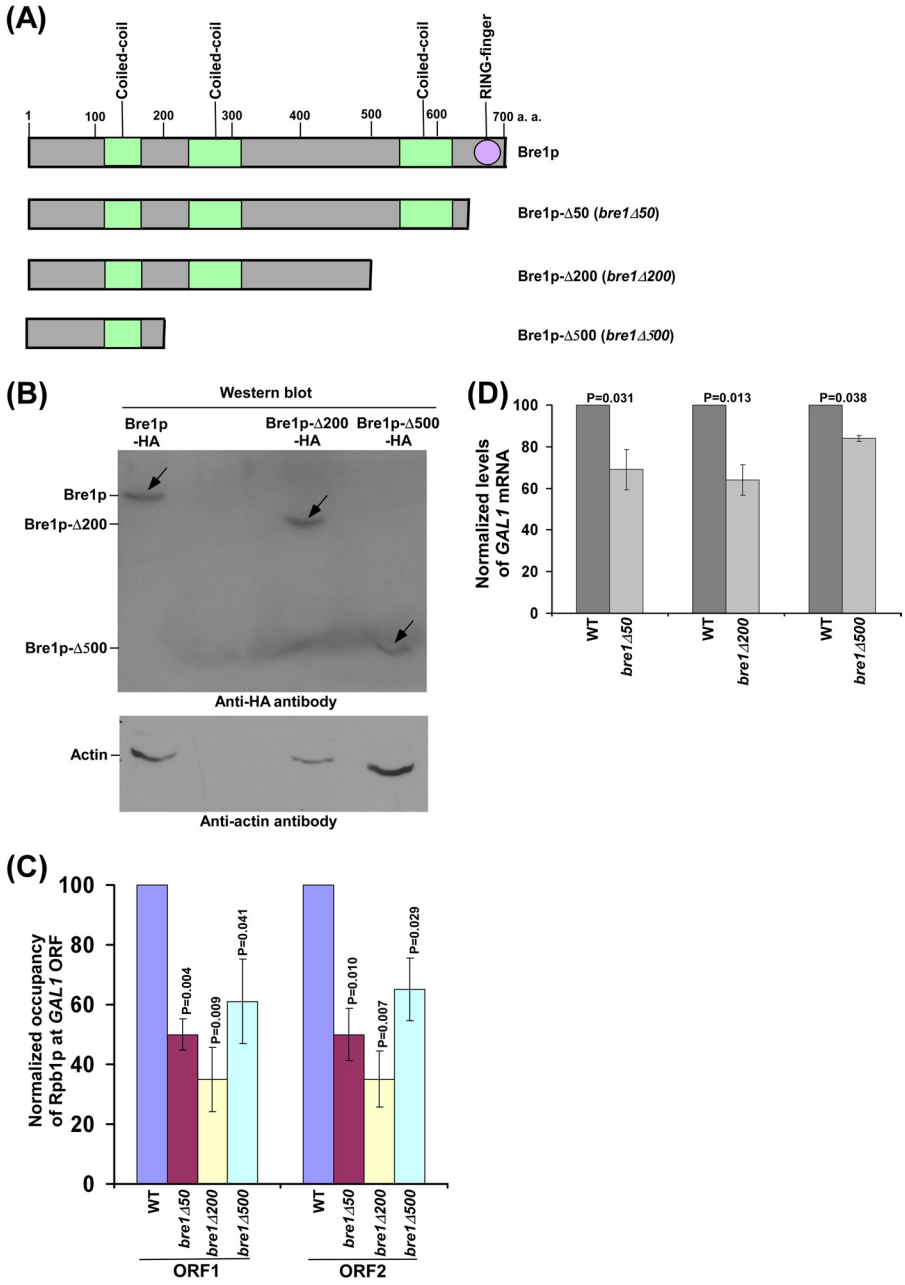

FIGURE 5. **A non-RING domain of Bre1p represses the association of RNA polymerase II with *GAL1*.** *A,* schematic diagram of different Bre1p mutants. *B,* Western blot analysis. *C,* analysis of RNA polymerase II association with *GAL1* in different Bre1p mutants. Yeast strains (RSY12, RSY13, RSY14, and RSY15) were grown, cross-linked, and immunoprecipitated as described in the legend to Fig. 1*B*. The *p* values for the differences between wild-type and Bre1p mutants are presented on *top* of the histograms of the mutants. *D,* analysis of *GAL1* transcription by RT-PCR. Yeast cells were grown as described in the legend to Fig. 1*B*.

role in association of RNA polymerase II with the active gene, and thus transcription.

Functional Analysis of the RING and Non-RING Domains of Bre1p in Association of RNA Polymerase II with GAL10, ADH1, and RPS5, and hence Transcription—So far, we have demonstrated that the absence of the RING finger with/without a part of the non-RING domain of Bre1p impairs the association of RNA polymerase II with *GAL1* (and hence transcription), similar to the Δ*rad6* and H2B K123R mutants. However, deletion of the whole *BRE1* reverses the phenotypes caused by loss of the RING finger with/without a portion of non-RING domain at *GAL1*. These results support the repressive role of a non-RING domain of Bre1p in association of RNA polymerase II with *GAL1* (and hence transcription), as discussed above. To determine whether similar results are found at other genes, we extended our studies to another galactose-inducible gene, *GAL10*. Similar to the results at *GAL1*, we found that association of RNA polymerase II with *GAL10* (and hence transcription) was significantly impaired in the Δ*rad6* and H2B K123R mutant strains (Fig. 6, *A–C* and *E*). On the other hand, the association of RNA polymerase II with *GAL10* (and hence transcription) was not altered in the Δ*bre1* strain in comparison to the wild-type equivalent (Fig. 6, *D* and *E*), as observed at *GAL1*. However, deletion of the RING finger of Bre1p and adjacent coiled-coil domain significantly impaired the association of RNA polymerase II with *GAL10* (Fig. 6*F*), and consistently,

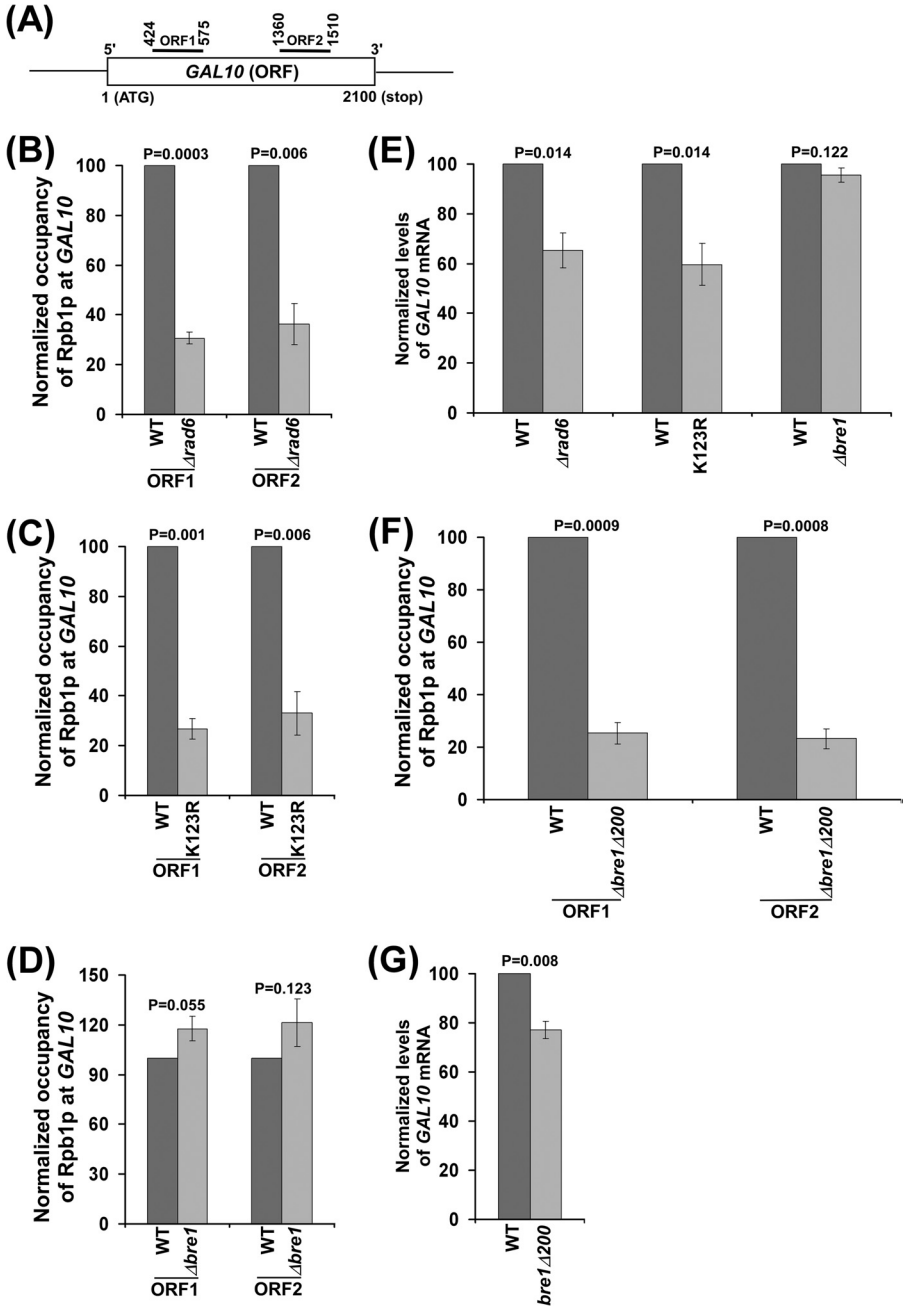

FIGURE 6. **Analysis of the association of RNA polymerase II with *GAL10*.** *A,* a schematic diagram showing the locations of the primer pairs (ORF1 and ORF2) at the *GAL10* coding sequence for ChIP analysis. The *numbers* are presented with respect to the position of the first nucleotide of the initiation codon (+1). *B,* Rad6p promotes the association of RNA polymerase II with *GAL10*. Yeast strains (STY1 and STY2) were grown, cross-linked, and immunoprecipitated as described in the legend to Fig. 1*B*. *C,* association of RNA polymerase II with *GAL10* is significantly decreased in the histone H2B-K123R point mutant strain. Yeast strains (YKH045 and YKH046) were grown, cross-linked, and immunoprecipitated as described in the legend to Fig. 1*B*. *D,* association of RNA polymerase II with *GAL10* is not impaired in the absence of Bre1p. Yeast strains (YTT31 and SLY1a) were grown, cross-linked, and immunoprecipitated as described in the legend to Fig. 1*B*. *E,* analysis of *GAL10* transcription by RT-PCR. Yeast strains were grown as described in the legend to Fig. 1*B* prior to harvesting for mRNA analysis. *F* and *G,* analysis of RNA polymerase II association with *GAL10* and transcription in the *bre1Δ200* and wild-type strains. Both wild-type and mutant strains were grown as described in the legend to Fig. 1*B*.

GAL10 transcription was also impaired (Fig. 6*G*). Thus, like the results at *GAL1*, we found that the RING finger and adjacent coiled-coil domain of Bre1p promoted association of RNA polymerase II with *GAL10*, and hence transcription. However, deletion of the whole *BRE1* reversed the phenotype of the RING finger and adjacent coiled-coil domain at *GAL10* (Fig. 6, *D–G*). We also found similar results at non-*GAL* genes, such as *ADH1* and *RPS5* (Fig. 7, *A–D*). Thus, a non-RING domain of Bre1p has a repressive role in association of RNA polymerase II with active genes and hence transcription.

DISCUSSION

Bre1p is an E3 ubiquitin ligase for histone H2B ubiquitylation that promotes the association of RNA polymerase II with the coding sequence of active gene and hence transcriptional elongation (8–12). Therefore, the deletion of *BRE1* is expected to

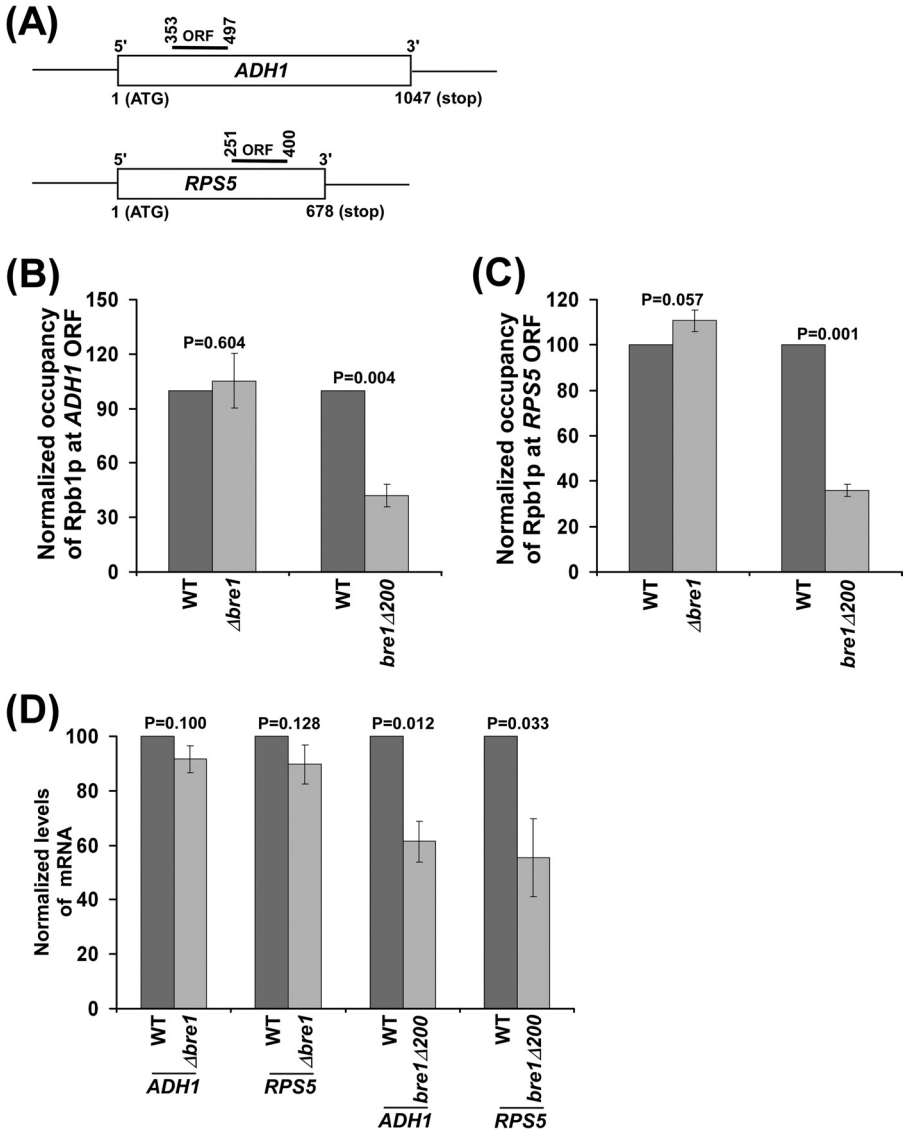

FIGURE 7. **Analysis of the association of RNA polymerase II with *ADH1* and *RPS5*.** *A,* the schematic diagrams showing locations of the primer pairs at the *ADH1* and *RPS5* coding sequences for the ChIP analysis. The *numbers* are presented with respect to the position of the first nucleotide of the initiation codon (+1). *B* and *C,* association of RNA polymerase II with *ADH1* and *RPS5* is altered in the *bre1Δ200* strain, but not in the Δ*bre1* strain. Wild-type and mutant strains were grown in YPD up to an A_{600} of 1.0 prior to cross-linking. Immunoprecipitations were performed as described in the legend to Fig. 1*B*. *D,* RT-PCR analysis of *ADH1* and *RPS5* mRNAs. Yeast strains were grown as in *panel B*.

impair the association of RNA polymerase II with the active coding sequence as well as transcription, similar to the Δ*rad6* and H2B-K123R mutant strains. Surprisingly, we find here that deletion of *BRE1* does not impair the association of RNA polymerase II with the coding sequence of *GAL1* following transcriptional induction (Fig. 1, *D* and *F*). Consistently, *GAL1* transcription is not altered in the Δ*bre1* strain (Fig. 1, *E* and *G*). On the other hand, the association of RNA polymerase II with the active *GAL1* coding sequence is significantly impaired in Δ*rad6* and H2B-K123R mutant strains (Fig. 1, *B*, *C,* and *E*). Similar results are also obtained at several other genes such as *GAL10*, *ADH1*, and *RPS5* (Figs. 6 and 7). These results raise the possibility that there may be a redundant gene that has compensated the function of Bre1p in the Δ*bre1* strain. However, we rule out this possibility, based on previous studies (15, 16, 19) and our studies (Fig. 1*H*) that demonstrated a dramatic loss of histone H2B ubiquitylation in the Δ*bre1* strain. Furthermore, we find that the Δ*bre1* strain is sensitive to genotoxic agents, such as MMS and HU, similar to the Δ*rad6* strain (Fig. 1*I*). Moreover, Rad6p does not complement the function of Bre1p in the Δ*bre1* strain as there is a drastic impairment of histone H2B ubiquitylation in the Δ*bre1* strain (Fig. 1*H*) (15, 16, 19). Therefore, there does not appear to be a gene that is redundant to Bre1p for histone H2B ubiquitylation in yeast. Thus, the invariant association of RNA polymerase II as well as transcription in the Δ*bre1* strain support the repressive role of Bre1p in addition to its stimulatory function (via H2B ubiquitylation) in association of RNA polymerase II with active gene and hence transcription, as schematically shown in Fig. 8.

Although complete deletion of *BRE1* does not impair the association of RNA polymerase II with active genes (as well as transcription), loss of the RING finger of Bre1p and a portion of the non-RING domain significantly reduces RNA polymerase II association and hence transcription (Figs. 2, *B* and *C*, and 5, *C*

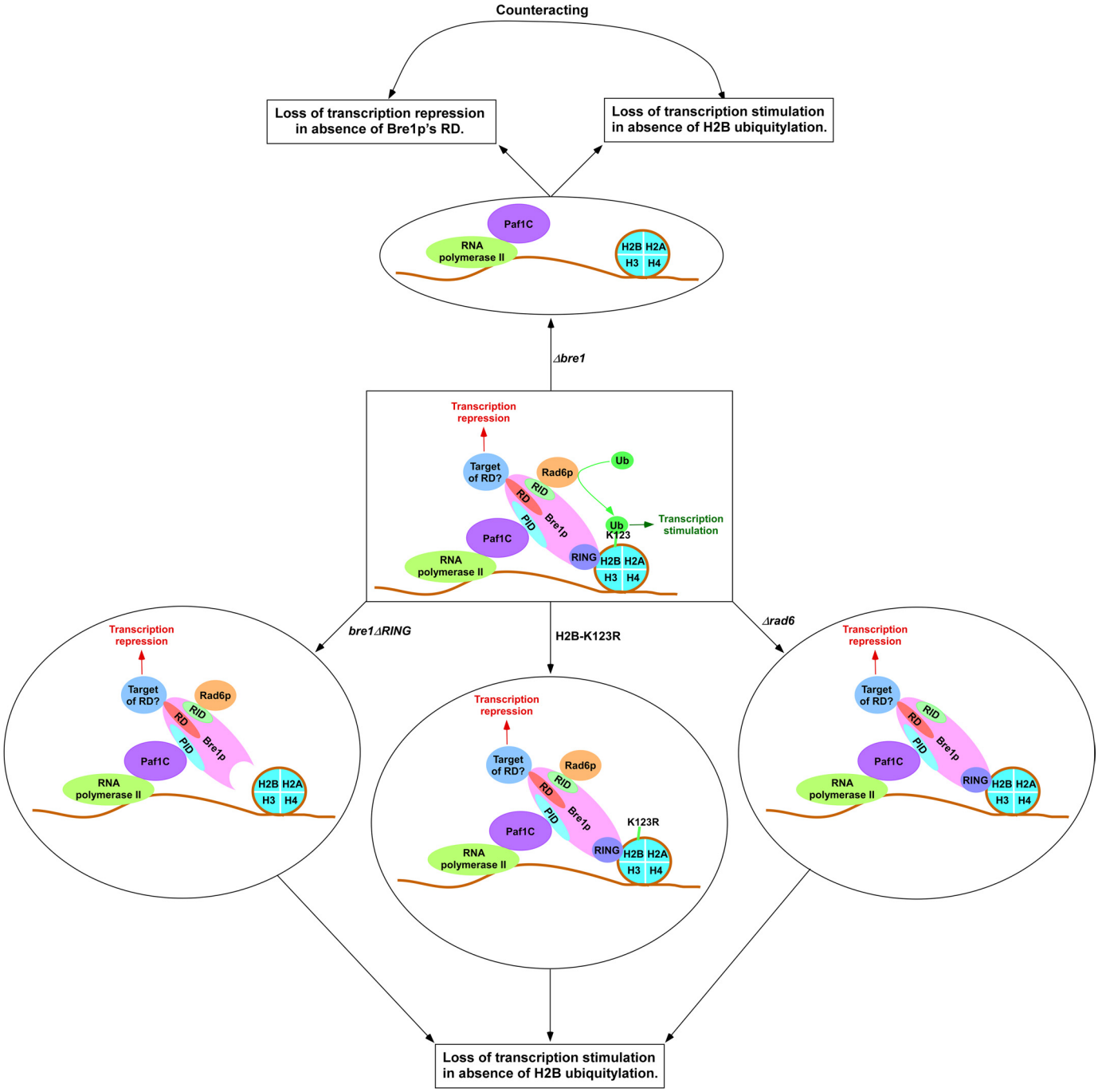

FIGURE 8. **The schematic diagram showing the stimulatory and repressive roles of Bre1p in transcription.** Bre1p interacts with chromatin via its RING domain at the C-terminal (19). Rad6p interacts with a domain within the first 210 amino acids at the N-terminal (19), and leads to targeted histone H2B ubiquitylation (19). Paf1C interacts with Bre1p (19) via its non-RING domain to promote histone H2B ubiquitylation activity of Rad6p-Bre1p (19, 34). Thus, *bre1ΔRING*, Δ*paf1*, Δ*rad6*, and H2B-K123R mutant strains impair histone H2B ubiquitylation (9, 15–17, 19), RNA polymerase II association, and transcription (8–12; this study). The non-RING part of Bre1p has a repression domain (*RD*) that lowers the association of RNA polymerase II with the active gene and hence transcription (this study), possibly via interaction with an unknown factor. The transcription stimulation is lost in the absence of histone H2B ubiquitylation in the *bre1ΔRING*, Δ*rad6*, and H2B-K123R mutant strains. When the whole *BRE1* is deleted, transcription repression is lost in the absence of the repression domain of Bre1p, in addition to impairment of transcriptional stimulation. These two opposing activities counteract, and hence the defect in transcription (or RNA polymerase II association) is not apparently observed in the Δ*bre1* strain. The *bre1Δ50* mutant is represented by *bre1ΔRING* (*i.e.* Bre1p without RING domain). *PID*, Paf1C interaction domain; *RID*, Rad6p interaction domain; *Ub*, ubiquitin.

and *D*), similar to the Δ*rad6* and H2B K123R mutant strains (Fig. 1, *B*, *C*, and *E*). Thus, the repressive role of Bre1p appears to be confined within a region toward the N-terminal in its non-RING domain. Deletion of the RING domain does not alter the interaction of Bre1p with Rad6p (19), but impairs the targeted histone H2B ubiquitylation (19) (Fig. 1*H*). Furthermore, the RING domain has been implicated to interact with histone H2B (19). Thus, the RING domain of Bre1p is essential for targeted histone H2B ubiquitylation via its interaction with histone H2B (Fig. 8). The first 210 amino acids at the N-terminal of Bre1p interact with Rad6p for histone H2B ubiquitylation (19). Because the N-terminal region of the non-RING domain of

Bre1p interacts with Rad6p for histone H2B ubiquitylation, the non-RING region at the N-terminal of Bre1p also has a transcriptional stimulatory role via histone H2B ubiquitylation in addition to its repressive role (Fig. 8).

Intriguingly, Paf1C that promotes histone H2B ubiquitylation activity of Rad6p-Bre1p (34) has recently been shown to interact biochemically with Bre1p (19). Consistent with recent biochemical data (19), we find here that the recruitment of Paf1C is significantly impaired in the Δ*bre1* strain (Fig. 4*B*). Our data further reveal that the RING domain of Bre1p is dispensable for recruitment of Paf1C (Fig. 4*F*). Thus, Paf1C appears to interact with the non-RING domain of Bre1p to promote histone H2B ubiquitylation. Therefore, the association of RNA polymerase II with *GAL1* (as well as transcription) is significantly decreased in the Δ*paf1* strain (Fig. 4, *D* and *E*), similar to Δ*rad6*, H2B K123R, and *Bre1Δ50* mutant strains (Figs. 1, *B, C,* and *E,* and 2, *B* and *C*). Taken together, our results support that the non-RING domain of Bre1p plays a crucial role to promote histone H2B ubiquitylation via its interaction with Paf1C and Rad6p in addition to its repressive function as schematically shown in Fig. 8.

It has been recently demonstrated that deletion of the RING domain of Bre1p does not alter its interaction with Rad6p (19). Furthermore, as discussed above, Paf1C interacts with the non-RING domain of Bre1p, and its recruitment is not impaired in the absence of the RING domain of Bre1p (Fig. 4*F*). Thus, deletion of the RING domain of Bre1p does not direct the degradation or disassembly of the entire complex (Fig. 8). The complete deletion of *BRE1* would dissociate the complex as Rad6p and Paf1C would loose their interaction with Bre1p (Fig. 8). However, the individual subunits following disintegration of the complex do not display residual activity toward histone H2B ubiquitylation as several previous studies (15, 16, 19) and our current studies (Fig. 1*H*) have demonstrated a dramatic loss of histone H2B ubiquitylation in the Δ*bre1* strain. Thus, reversal of the defect of RNA polymerase II association with *GAL1* caused by the loss of the RING domain in the Δ*bre1* strain is not mediated by the residual activity of the individual component, but rather a repression domain of Bre1p as schematically shown in Fig. 8.

As discussed above, the complete deletion of *BRE1* lowers the recruitment of Paf1C to *GAL1* (Fig. 4*B*) and the absence of Paf1C impairs the association of RNA polymerase II with *GAL1* as well as transcription (Fig. 4, *D* and *E*). Based on these results, it is expected to observe an impairment of RNA polymerase II association with *GAL1*, and transcription in the absence of Bre1p. However, we find that RNA polymerase II association with *GAL1* is not impaired in the Δ*bre1* strain (Fig. 1, *D* and *F*). Consistently, *GAL1* transcription is not decreased in the absence of Bre1p (Fig. 1, *E* and *G*). These results support the presence of a repression domain within the non-RING domain of Bre1p, as discussed above. Such a domain might be exhibiting its repressive role by interacting with another factor(s). The loss of interaction of yet another unknown factor(s) with Bre1p in the Δ*bre1* strain might have reversed the defect in RNA polymerase II association and transcription in the absence of the RING domain and interactions of Bre1p with Rad6p and Paf1C in Δ*bre1*, as schematically shown in Fig. 8. Intriguingly, protein-protein interaction studies (18, 54–56) revealed the interaction of Bre1p with histone deacetylase, a transcriptional repressor. Thus, histone deacetylase might be repressing transcription via its interaction with Bre1p. Such repression is relieved in the absence of Bre1p, and hence the defect in RNA polymerase II association with *GAL1* in the absence of the RING domain (or histone H2B ubiquitylation) is reversed in the null mutation of *BRE1* (Fig. 8). Alternatively, Bre1p might be interacting with other transcriptional repressor(s). However, these possibilities remain to be further elucidated. Nonetheless, our data demonstrate that a non-RING domain of Bre1p has a repressive role, and thus, the complete deletion of *BRE1* reverses the defect in transcription and RNA polymerase II association phenotypes caused by the loss of H2B ubiquitylation or the RING domain (or interactions with Rad6p and Paf1C), as schematically shown in Fig. 8.

Recently, the homologue of Bre1p in humans has been shown to repress the expression of the proto-oncogenes via inhibition of the recruitment of TFIIS that promotes transcription (48). Based on these recent results, it is expected that Bre1p in yeast might be exhibiting its repressive role by inhibiting the recruitment of TFIIS. However, our data reveal that Bre1p does not alter the recruitment of TFIIS in yeast (Fig. 3, *B* and *C*). Thus, the function of the homologue of Bre1p in humans in repressing the expression of proto-oncogenes via TFIIS appears to be gene-specific or more complex as compared with yeast.

In summary, we find that the RING domain of Bre1p has a stimulatory role in targeted histone H2B ubiquitylation for enhanced transcription as well as RNA polymerase II association with the active gene (Fig. 8). On the other hand, the non-RING domain of Bre1p has both the stimulatory and repressive roles in regulating the association of RNA polymerase II with the active gene and hence transcription (Fig. 8). The stimulatory function of the non-RING domain of Bre1p is mediated via its interaction with Rad6p and Paf1C, which promotes H2B ubiquitylation (Fig. 8). Although the function of Bre1p in transcription stimulation is well known via histone H2B ubiquitylation, this study unravels for the first time a new repressive role of the non-RING domain of Bre1p in regulating the association of RNA polymerase II with active gene and hence transcription (Fig. 8). Such a repressive role appears to be confined within the first ~200 amino acids at the N-terminal of Bre1p. This domain of Bre1p may be exhibiting its repressive role by decreasing the histone H2B ubiquitylation activity. However, we rule out this possibility as a recent study has implicated the interaction of this domain with Rad6p for histone H2B ubiquitylation (19). Consistently, deletion of this domain has been shown to impair histone H2B ubiquitylation (19). Furthermore, removal of this domain does not alter Bre1p homomeric complex formation (19). Thus, the first ~200 amino acid domain of Bre1p is likely to play its repressive role via a different mechanism. It has been recently demonstrated that the 1–210-amino acid domain of Bre1p is ubiquitylated by Rad6p (19), and such ubiquitylation may play a repressive role in association of RNA polymerase II and transcription. Alternatively, this domain may also play a repressive role in association of RNA polymerase II via histone deacetylation, because Bre1p has been shown to interact with histone deacetylase (18, 54–56), as discussed above. However,

these possibilities remain to be further elucidated. Nonetheless, this study demonstrates for the first time a new repressive role of Bre1p in regulation of RNA polymerase II association with active gene and hence transcription. Such a repressive role of Bre1p is likely to play a crucial function to slow down elongating RNA polymerase II for allowing histone H2B ubiquitylation and subsequent chromatin reassembly in maintaining productive transcription, because previous studies have implicated histone H2B ubiquitylation in chromatin reassembly in the wake of elongating RNA polymerase II (10, 11). Thus, the repressive role of Bre1p appears to be physiologically linked to its stimulatory function in histone H2B ubiquitylation, chromatin dynamics, and transcription.

Acknowledgments—We thank Mary Ann Osley, Ali Shilatifard, David Stillman, and Judith K. Davie for yeast strains; and Shivani Malik for critical reading of this manuscript.

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