Rad6 plays a role in transcriptional activation through ubiquitylation of histone H2B

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Covalent modifications of the histone N tails play important roles in eukaryotic gene expression. Histone acetylation, in particular, is required for the activation of a subset of eukaryotic genes through the targeted recruitment of histone acetyltransferases. We have reported that a histone C tail modification, ubiquitylation of H2B, is required for optimal expression of several inducible yeast genes, consistent with a role in transcriptional activation. H2B was shown to be ubiquitylated and then deubiquitylated at the *GAL1* core promoter following galactose induction. We now show that the Rad6 protein, which catalyzes monoubiquitylation of H2B, is transiently associated with the *GAL1* promoter upon gene activation, and that the period of its association temporally overlaps with the period of H2B ubiquitylation. Rad6 promoter association depends on the Gal4 activator and the Rad6-associated E3 ligase, Bre1, but is independent of the histone acetyltransferase, Gcn5. The SAGA complex, which contains a ubiquitin protease that targets H2B for deubiquitylation, is recruited to the *GAL1* promoter in the absence of H2B ubiquitylation. The data suggest that Rad6 and SAGA function independently during galactose induction, and that the staged recruitment of these two factors to the *GAL1* promoter regulates the ubiquitylation and deubiquitylation of H2B. We additionally show that both Rad6 and ubiquitylated H2B are absent from two regions of transcriptionally silent chromatin but present at genes that are actively transcribed. Thus, like histone H3 lysine 4 and lysine 79 methylation, two modifications that it regulates, Rad6-directed H2B ubiquitylation defines regions of active chromatin.

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Chromatin remodeling plays a key role in the regulation of eukaryotic gene expression through the activity of both ATP-dependent nucleosome remodeling factors and histone modifying activities (Belotserkovskaya and Berger 1999). Histone modifications such as acetylation, phosphorylation, and methylation occur on the core histone N tails, and they are frequently targeted to nucleosomes in the promoter regions of active genes (Grunstein 1997; Spencer and Davie 1999). These modifications are regulated by a large group of enzymes, often subunits of multiprotein factors that are themselves recruited to specific genes through interactions with site-specific DNA-binding proteins (Kuo et al. 2000; Peterson and Workman 2000; Bhaumik and Green 2001; Hassan et al. 2001; Larschan and Winston 2001). Although the histone N tails carry the majority of the modifications, the histone H2A and H2B C tails are also posttranslationally modified. One such C tail modification, ubiquitylation, has also been linked to transcription (Bohm et al. 1980; West and Bonner 1980; Hensold et al. 1988; Davie et al. 1991; Bradbury 1992; Jason et al. 2002). Early work showed that nucleosomes in the 5′ regulatory regions of highly transcribed genes frequently contained elevated levels of ubiquitylated histones H2A and H2B, suggesting that the modified histones play a role in transcriptional activation (Levinger and Varshavsky 1982; Barsoum and Varshavsky 1985; Nickel et al. 1989). In addition, recent studies have implicated ubiquitylated H2B in heterochromatic gene silencing and gene repression in yeast (Briggs et al. 2002; Dover et al. 2002; Ng et al. 2002; Sun and Allis 2002). However, the role of histone ubiquitylation in transcription is poorly understood. Because histones are primarily monoubiquitylated, which is not associated with protein turnover, the presence of ubiquitylated histones in chromatin has been postulated to confer a structural or signaling role (Briggs et al. 2002; Henry and Berger 2002; Jason et al. 2002; Sun and Allis 2002).

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Histone H2B is the only histone species in *Saccharomyces cerevisiae* currently known to be ubiquitylated (Swerdlow et al. 1990; Robzyk et al. 2000). Between 1 and 10% of total H2B in this organism is modified by the attachment of a single ubiquitin moiety, although this number could be higher if ubiquitin conjugation is a highly dynamic process as has been suggested (Busch and Goldknopf 1981; Seale 1981; Wu et al. 1981; Mueller et al. 1985; Robzyk et al. 2000; Sun and Allis 2002; Henry et al. 2003). The evolutionarily conserved ubiquitin conjugating enzyme, Rad6/Ubc2, catalyzes monoubiquitylation of H2B in yeast (Jentsch et al. 1987; Sung et al. 1988; Robzyk et al. 2000). Rad6 is a multifunctional protein with roles in postreplication DNA damage repair, meiosis, transcriptional silencing, and protein turnover (Montelone et al. 1981; Borts et al. 1986; Dohmen et al. 1991; Huang et al. 1997; Hoege et al. 2002; Turner et al. 2002). Its role in meiosis and transcriptional silencing appears to be directly related to its role in ubiquitylation of H2B because mutation of *RAD6* or loss of the H2B ubiquitylation site confer similar phenotypes in these two processes (Huang et al. 1997; Robzyk et al. 2000; Dover et al. 2002; Ng et al. 2002; Sun and Allis 2002). However, Rad6 targets a number of other substrates for modification besides H2B, and its substrate specificity appears to be determined by its association with different E3 ubiquitin ligases (Kornitzer et al. 1994; Kaplun et al. 2000; Pickart 2001; Hoege et al. 2002). Until recently, there was no known E3 that specified H2B ubiquitylation by Rad6. The Bre1 protein, a RING finger protein with structural similarities to other E3 ubiquitin ligases, has emerged as an E3 that directs Rad6 to H2B (Joazeiro and Weissman 2000; Hwang et al. 2003; Wood et al. 2003). Deletion of *BRE1* globally reduces ubiquitylated H2B levels in yeast and confers several phenotypes that are similar to the loss of the H2B ubiquitylation site. Thus, Bre1 is predicted to play a role in the same cellular processes that are regulated by H2B ubiquitylation.

H2B ubiquitylation has been linked to transcription in yeast through its role in the *trans*-histone regulation of histone H3 lysine 4 and lysine 79 methylation (Briggs et al. 2002; Dover et al. 2002; Ng et al. 2002; Sun and Allis 2002). Both of these H3 methylation marks have been associated with transcriptionally active euchromatin, and their presence is reduced in regions of silent chromatin (Strahl et al. 1999; Bernstein et al. 2002; Santos-Rosa et al. 2002; van Leeuwen et al. 2002; Ng et al. 2003a). Moreover, a genomewide analysis of H3 lysine 4 methylation showed that lysine 4 trimethylation is concentrated at the 5′ regions of transcriptionally active genes (Ng et al. 2003b). Given the role of H2B ubiquitylation in regulating H3 lysine 4 methylation, this suggested that the ubiquitin modification would also play a role in active transcription. In support of this view, we recently reported that monoubiquitylation of H2B is required for the optimal expression of two highly inducible yeast genes, *GAL1* and *SUC2* (Henry et al. 2003). Upon induction of the *GAL1* gene, H2B was found to be sequentially ubiquitylated and then deubiquitylated at the *GAL1* core promoter prior to the significant accumulation of transcripts. Deubiquitylation was shown to be controlled by the activity of the ubiquitin protease, Ubp8, a stoichiometric subunit of the SAGA coactivator, which is one of the earliest factors recruited to the *GAL* promoter following galactose induction (Sanders et al. 2002; Bryant and Ptashne 2003). However, the requirements for H2B ubiquitylation were not defined in this study. In this report, we investigated the role of Rad6 in the ubiquitylation of H2B at the *GAL1* gene during galactose activation. We found that, like SAGA, Rad6 was also recruited to the *GAL1* promoter, but, unlike SAGA, it remained there only transiently. Rad6 promoter association required the Gal4 activator and the Rad6-associated E3, Bre1, and thus in addition to its role in DNA damage repair, Rad6 can also be classified as a transcriptional coactivator. The period of Rad6 promoter association closely paralleled the period of H2B ubiquitylation and appeared to precede the recruitment of SAGA. This suggests that the staged recruitment of Rad6 and SAGA controls the ubiquitylation and deubiquitylation of H2B at the *GAL* promoter. We also show that H2B ubiquitylation, like the two H3 methylation marks that it controls, is generally excluded from silent chromatin but present in regions of transcriptionally active euchromatin. Thus, H2B ubiquitylation can also be considered as a mark of active chromatin.

Results

Rad6 is transiently associated with the GAL1 promoter

The *GAL* genes provide an excellent model for gene activation in eukaryotes. The genes are repressed in glucose-containing medium and induced when cells are shifted to the presence of galactose (Johnston and Carlson 1992; Trumbly 1992; Johnston et al. 1994). Upon gene activation, repression by Mig1/Tup1-Ssn6 is first relieved, and then a cascade of transcription factor recruitment to the *GAL* promoters is initiated by the enhanced binding of the Gal4 activator to UAS sites in each promoter. Studies performed with the *GAL1-GAL10* genes have shown that one of the earliest factors to be recruited is the SAGA histone acetyltransferase complex, which requires Gal4 for its association with the *GAL* promoter (Bhaumik and Green 2001; Larschan and Winston 2001; Bryant and Ptashne 2003). Subsequently, Mediator, TBP, and RNA polymerase II become associated with the core promoter, and transcription initiates (Bryant and Ptashne 2003).

In addition to these factors, we recently showed that H2B ubiquitylation contributes to the maximal transcription of the *GAL* genes (Henry et al. 2003). In the absence of this histone modification, the levels of *GAL1* transcript were reduced following galactose activation. Consistent with a role in the initiation of *GAL1* transcription, H2B was found to be transiently ubiquitylated at the *GAL1* core promoter at a time that preceded the accumulation of *GAL1* mRNA. We therefore asked if Rad6, which directs monoubiquitylation of H2B, was also recruited to the *GAL* promoter upon gene induction

using the method of chromatin immunoprecipitation (ChIP; Orlando and Paro 1993; Hecht et al. 1996; Kuo and Allis 1999). ChIP was carried out during growth in glucose medium and at 30-min intervals after a shift to galactose medium in a yeast strain that contained an HA epitope-tagged *RAD6* gene (Fig. 1A). The data showed that Rad6 was dynamically associated with the *GAL1* UAS. Maximal occupancy occurred at 30–60 min after the shift to galactose, and by 120 min Rad6 binding to the *GAL1* UAS was substantially reduced. The peak of association significantly preceded the appearance of *GAL1* transcripts as measured by Northern blot analysis (Fig. 1A, inset), suggesting that Rad6 recruitment to the UAS regulates a very early event in *GAL1* transcription. This event is most likely the Rad6-mediated ubiquitylation of H2B, as the period of Rad6 association with the UAS and the period of H2B ubiquitylation at the core promoter almost exactly coincided (Fig. 1B).

Next, we determined the genetic requirements for Rad6 association with the *GAL1* promoter. We first asked if Rad6 recruitment occurred in an *htb1-K123R* mutant, where H2B is not ubiquitylated (Fig. 1C). In this mutant, Rad6 also transiently associated with the *GAL* promoter, although the peak of association was delayed by ~30 min and the drop in promoter binding occurred somewhat more slowly. Thus, prior ubiquitylation of H2B is not a prerequisite for Rad6 promoter association, although it may control the kinetics of Rad6 recruitment and departure from the promoter. Because the Gal4 activator interacts in vivo and in vitro with a number of chromatin remodeling/modifying activities and is required to bring SAGA to the *GAL1* promoter in vivo (Melcher and Johnston 1995; Bhaumik and Green 2001; Larschan and Winston 2001; Carrozza et al. 2002), we next asked if it was also required to bring Rad6 to *GAL1*. ChIP was performed in a *gal4*Δ strain, and the results showed that Rad6 did not occupy the *GAL1* promoter when the activator was absent (Fig. 2A). Rad6 was re-

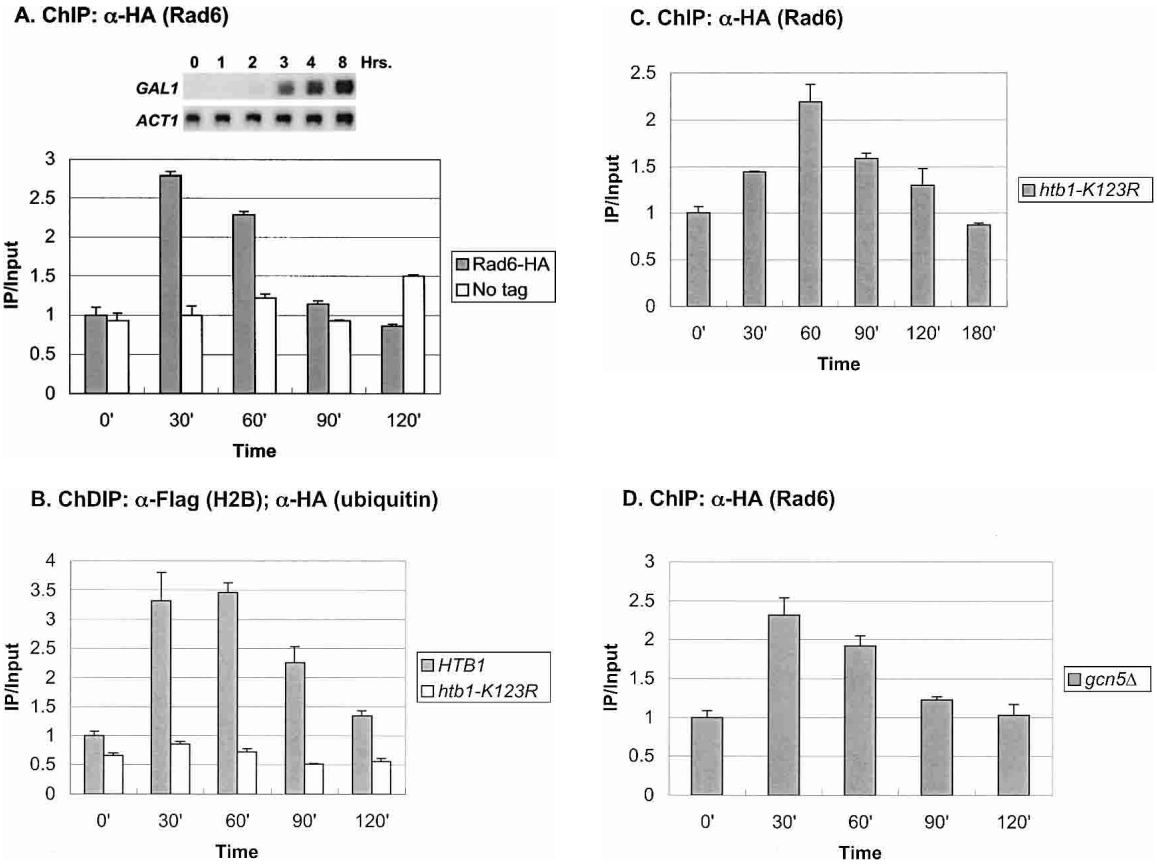

Figure 1. Rad6 is transiently associated with the *GAL1* promoter. (*A*) Strains YKH010 (Rad6-HA *HTB1*) and JR5-2A (No tag control *HTB1*) were grown in YPD medium and shifted to YP + 2% galactose medium, and chromatin immunoprecipitation (ChIP) was performed with anti-HA antibodies at the indicated times. PCR analysis in real time was used to measure the abundance of *GAL1* UAS sequences in immunoprecipitated (IP) DNA relative to input DNA. The Northern blot *inset* is from Figure 7A. (*B*) Strains YKH045 (*Flag-HTB1*; HA-ubiquitin) and YKH046 (*Flag-htb1-K123;* HA-ubiquitin) were grown as described in panel *A*. Chromatin double immunoprecipitation (ChDIP) was sequentially performed with anti-Flag and anti-HA antibodies, and PCR in real time was used to measure the abundance of *GAL1* core promoter sequences in the IP DNA (α-HA) relative to input DNA (α-Flag). The data were normalized to the IP/Input ratios for *INT-V*, which is not regulated by galactose and thus served as a control. (*C,D*) Strains YKH017 (Rad6-HA *htb1-K123R*) and YCH001 (Rad6-HA *HTB1 gcn5*Δ) were grown as described in panel *A*, and ChIP was performed with anti-HA antibodies, followed by PCR analysis in real time using primers that detect the *GAL1* UAS sequences.

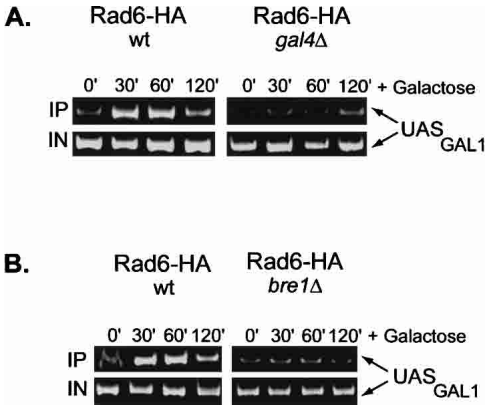

Figure 2. Rad6 recruitment to the *GAL1* promoter requires the Gal4 activator and the Bre1 E3 ligase. After a shift to YP + galactose medium, ChIP and conventional PCR analysis were carried out in a *RAD6-HA HTB1* strain that contained wild-type genes and (*A*) *gal4*Δ (YCH001) or (*B*) *bre1*Δ (YCH002) deletions. The PCR reactions were electrophoresed on a 10% acrylamide gel, which was stained with ethidium bromide. All reactions were performed in the linear range of amplification.

cently reported to be in a complex with Bre1, an E3 ligase that directs Rad6 to monoubiquitylate H2B and is required for the presence of Rad6 at the constitutive *PMA1* promoter (Hwang et al. 2003; Wood et al. 2003). The binding of Rad6 to the *GAL1* UAS was also significantly reduced in a *bre1*Δ mutant, indicating that a Rad6–Bre1 complex is likely to be recruited to *GAL1* (Fig. 2B). Finally, Rad6 continued to show transient occupancy of the *GAL1* promoter in a *gcn5*Δ mutant, indicating that histone acetylation is also not required for its recruitment (Fig. 1D). Together, the results suggest that in response to galactose activation, the Gal4 activator recruits the Rad6–Bre1 complex to the *GAL1* UAS, where it results in the ubiquitylation of H2B at the core promoter. BEcause we have shown that H2B ubiquitylation is important for the maximal transcription of *GAL1* (Henry et al. 2003), the data indicate that Rad6, like SAGA, functions as a transcriptional coactivator at the *GAL1* gene.

SAGA and Rad6 show different patterns of GAL1 promoter association

The SAGA complex is one of the earliest factors recruited to the *GAL* genes following galactose induction, and it has two known roles at the *GAL1* core promoter (Bryant and Ptashne 2003). First, the Ubp8 subunit, a ubiquitin protease, targets H2B for deubiquitylation (Henry et al. 2003), and second, the Spt3 subunit is required for recruitment of the TATA-binding protein, TBP (Dudley et al. 1999; Bhaumik and Green 2001, 2002; Larschan and Winston 2001). Because deubiquitylation of H2B closely follows its ubiquitylation, we compared the recruitment of SAGA to that of Rad6 following a shift from glucose to galactose medium. As measured by ChIP in a strain containing a Gcn5-HA protein, SAGA showed increased association with the *GAL1* UAS over the time course of the experiment (Fig. 3A), similar to the pattern we recently reported (Henry et al. 2003). At 120 min after the shift to galactose medium, a time when Rad6 was no longer associated with the promoter (Fig. 1A), SAGA was almost four times more concentrated at the *GAL1* UAS than it was in glucose medium. Thus, the two coactivators show very different patterns of association with the *GAL1* promoter: Rad6 binds transiently, whereas SAGA remains bound once it is recruited. To determine if Rad6 binds before SAGA, we examined the promoter association of the two factors at shorter time intervals following a shift from raffinose to galactose medium, which leads directly to *GAL1* activation (Fig. 3C). The data show that Rad6 was again transiently associated with the *GAL1* promoter. Moreover, Rad6 reached its maximal occupancy of the UAS earlier than SAGA (15–30 min vs. 30–90 min), supporting the view that Rad6 may be the first factor recruited to the *GAL1* UAS upon galactose induction. As in the glucose-to-galactose shift, the period of Rad6 association preceded the maximal accumulation of *GAL1* transcripts, consistent with a role for Rad6 recruitment in transcriptional activation (data not shown). Finally, we asked if Rad6-directed ubiquitylation of H2B were required for SAGA association with the *GAL1* promoter by performing ChIP in an *htb1-K123R* mutant. SAGA continued to accumulate at the *GAL1* UAS in the absence of both the H2B ubiquitylation site and the Bre1 E3 ligase (Fig. 3A; data not shown). As previously noted, Gcn5-associated H3 Lys 9/Lys14 acetylation levels did not change during *GAL1* induction (Bhaumik and Green 2001; Larschan and Winston 2001), and here we show that the *htb1-K123R* mutation has no effect on this histone modification (Fig. 3B). Thus, we propose that Rad6 precedes SAGA at the *GAL1* promoter, but that SAGA is recruited to the promoter independently of Rad6-mediated H2B ubiquitylation.

Histone H2B is transiently ubiquitylated at the PHO5 promoter

To determine if H2B ubiquitylation plays a role in the expression of other inducible genes, the levels of the modified histone were also examined during activation of the *PHO5* gene. This gene is regulated by an entirely different mechanism from *GAL1*, but like *GAL1*, *PHO5* transcription also shows a modest dependence on H2B ubiquitylation (see Fig. 7A, below; Vogel et al. 1989; Svaren and Horz 1997). Using the chromatin double immunoprecipitation assay, ubiquitylated H2B was found to transiently accumulate at the *PHO5* core promoter after phosphate was depleted from the medium (Fig. 4). The level of ubiquitylated H2B peaked ~2.3-fold above the repressed level at 90 min after the shift and declined to the uninduced level by 120 min, a time that preceded the maximal accumulation of *PHO5* transcripts. This rise strictly depended on the presence of the H2B ubiquitylation site and was specific to the *PHO5* gene, as the levels of ubiquitylated H2B remained unchanged at two

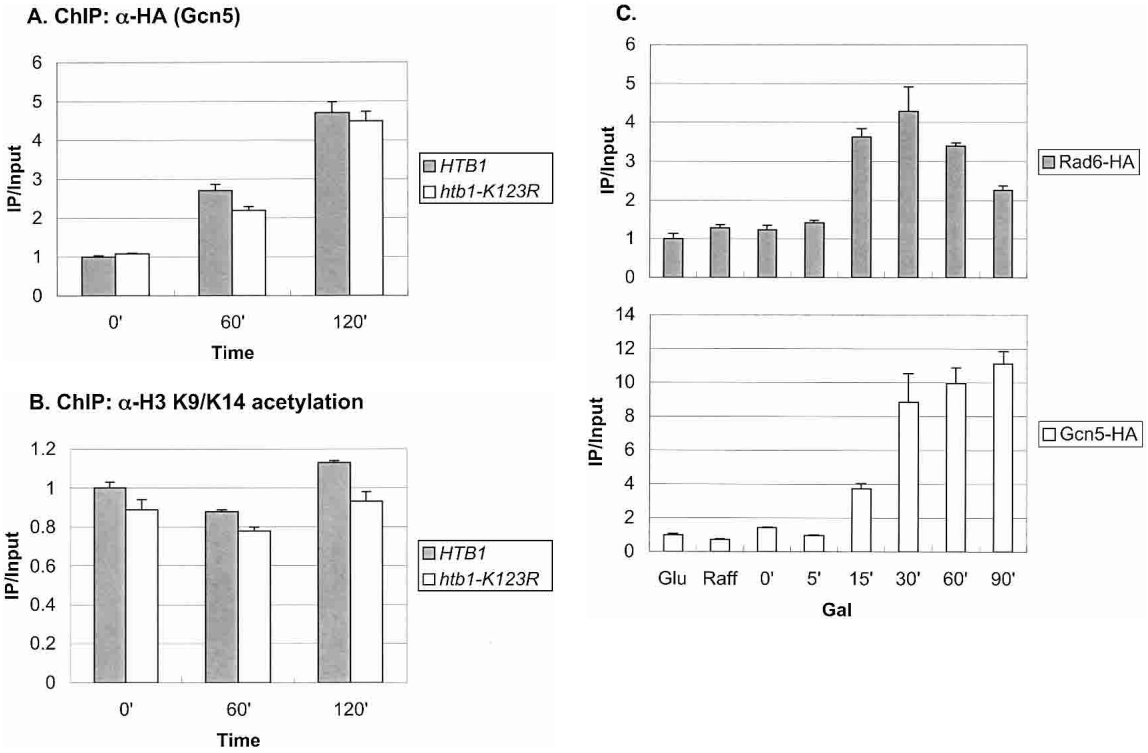

Figure 3. Rad6 and SAGA show different patterns of association with the *GAL1* promoter. (*A*) Strains carrying Gcn5-HA and either *HTB1* (YKH012) or *htb1-K123R* (YKH019) were subjected to ChIP and PCR analysis in real time as described in the legend to Figure 1A before and after a shift to YP + galactose medium. (*B*) ChIP was performed with anti-H3 K9/K14 acetylation antibodies in strains Y131 (*HTB1*) and Y133 (*htb1-K123R*) before and after a shift to YP + galactose medium, and the DNA was analyzed by PCR in real time to determine the levels of H3 acetylation at the *GAL1* core promoter. (*C*) ChIP was performed with anti-HA antibodies in strains YKH010 (Rad6-HA *HTB1*) and YKH0112 (Gcn5-HA *HTB1*) during growth in YPD (Glu), after 2 h in YP + 2% raffinose (Raff), and at the indicated times after galactose was added to a final concentration of 2%. PCR in real time was performed to measure the association of Rad6 and Gcn5 with the *GAL1* UAS. The data in each panel were normalized to the IP/Input ratio of the *INT-V* control sequences.

intergenic regions under the same growth conditions (Fig. 4; data not shown). Thus, the data suggest that H2B ubiquitylation and deubiquitylation may play general roles in the initiation of transcription following gene activation.

Distribution of H2B ubiquitylation

To determine if H2B ubiquitylation was restricted to transcribed genes, the levels of the modified histone were measured at different regions of the yeast genome by a chromatin double immunoprecipitation assay or ChDIP (Fig. 5A). Besides the induced *GAL1* and *PHO5* genes, two constitutively transcribed genes, *ACT1* and *PMA1*, also contained ubiquitylated H2B at their promoters, albeit at lower relative amounts. In addition to these transcribed genes, two ORF-free intergenic regions on chromosomes V and XVI contained relatively high levels of the H2B modification, whereas two regions of silenced chromatin, *TELVI-R* and *HMRa*, had the lowest levels of ubiquitylated H2B. This distribution is remarkably similar to the pattern of H3 Lys 79 methylation at the same genomic regions, which is dependent on H2B ubiquitylation (data not shown). Together, the results suggest that ubiquitylated H2B, like H3 Lys 4 and Lys 79 methylation, may be broadly distributed in transcriptionally active euchromatin and excluded from heterochromatin-like regions (Bernstein et al. 2002; Ng et al. 2003a,b). Next, we measured Rad6 association with the same genomic regions by ChIP (Fig. 5B). Like ubiquitylated H2B, Rad6 was present at the promoters of each of the induced or constitutive genes, but absent from the two regions of silent chromatin. Surprisingly, Rad6 was also absent from the two ORF-free regions, even though these regions contain relatively high levels of H2B ubiquitylation. This suggests that the ubiquitylated species may be differentially stabilized depending on its genomic location. For example, if the ORF-free regions are not transcribed, then there may be no targeted recruitment of a ubiquitin protease, such as occurs at the *GAL1* promoter upon SAGA association (Henry et al. 2003). Using similar reasoning, these data also imply that Rad6 may ubiquitylate H2B by both targeted and untargeted mechanisms.

We then asked how H2B ubiquitylation was distributed at an individual transcribed gene. We predicted that

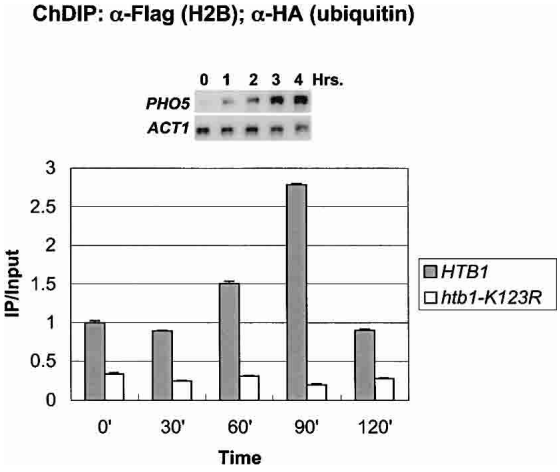

Figure 4. H2B is transiently ubiquitylated at the *PHO5* core promoter. ChDIP was carried out in strains YKH045 (*Flag-HTB1*) and YKH046 (*Flag-htb1-K123R*) during growth in YPD medium and at 30-min intervals after a shift to a medium lacking inorganic phosphate, and extracted DNA was analyzed by quantitative PCR in real time to determine the levels of H2B ubiquitylation at the *PHO5* core promoter. The data were normalized to the *INT-V* IP/Input ratios. The Northern blot *inset* is from Figure 7A.

it might be associated only with the core promoters of active genes, consistent with a role in transcription initiation. We addressed this question at the *GAL1* gene by performing ChDIP in glucose-repressed and galactose-induced cells, using PCR primers that corresponded to the *GAL1* core promoter, 5′ ORF, and 3′ ORF regions (Fig. 6A). Surprisingly, under repressed conditions, the 3′ ORF region was found to contain a significant level of ubiquitylated H2B, whereas the 5′ ORF region and core promoter had progressively lower levels of the modified histone. However, after 60 min of galactose induction, the level of ubiquitylated H2B rose disproportionately at the 5′ end of *GAL1*, resulting in an approximately equal distribution of the modified histone across the promoter and ORF. Thus, H2B ubiquitylation is, in fact, targeted to the 5′ end of *GAL1* upon gene induction, consistent with a role in transcription initiation, but its presence throughout the gene suggests that it might also play a role in transcription elongation. When the distribution of Rad6 was examined at the same *GAL1* regions by ChIP, little Rad6 was found to be associated with any region of the gene when cells were grown under repressing conditions (Fig. 6B). However, after galactose induction, ~2.5-fold more Rad6 was present at the *GAL1* UAS and core promoter as well as at the two ORF regions. This is consistent with Rad6 and ubiquitylated H2B having a role in both transcription initiation and transcription elongation.

H2B ubiquitylation plays an overlapping role with chromatin remodeling factors

Although our data indicate that H2B ubiquitylation might be fairly widespread in euchromatin, we have previously shown that the absence of the ubiquitylated species confers relatively modest effects on cell growth and gene expression (Henry et al. 2003). This suggests that the H2B modification might be functionally redundant with other histone modifying activities or nucleosome remodeling factors. In support of this view, we have noted two synthetic phenotypes in double mutants constructed between the *htb1-K123R* and *gcn5*Δ or *snf5*Δ alleles. First, *htb1-K123R snf5*Δ double mutants grow significantly more slowly than either single mutant, whereas *htb1-K123R gcn5*Δ double mutants show a Ts– growth defect (data not shown). Second, transcription of several inducible genes is severely compromised in the double mutants (Fig. 7). Whereas transcription of the *GAL1*, *PHO5*, and *SUC2* genes was reduced less than twofold in *htb1-K123R* single mutants (Fig. 7A), transcription of the SAGA-dependent *GAL1* and *PHO5* genes and the Swi/Snf-dependent *SUC2* gene was almost abolished in *htb1-K123R gcn5*Δ and *htb1-K123R snf5*Δ double mutants, respectively (Fig. 7B). This phenotype is unrelated to the growth defects of the double mutants because expression of the SAGA-dependent *INO1* gene and the Swi/Snf- and SAGA-dependent *HO* gene was unaffected in the same double mutant backgrounds (data not shown). Thus, H2B ubiquitylation becomes very important for the expression of specific genes in the absence of either Gcn5-dependent histone acetylation or ATP-dependent nucleosome remodeling, indicating that the H2B modification plays a functionally overlapping role with these two chromatin remodeling factors.

Discussion

In this study, we show that upon galactose induction, Rad6 is dynamically associated with the *GAL1* UAS, leading to a concomitant transient accumulation of ubiquitylated H2B at the core promoter. The temporal overlap between Rad6 promoter association and H2B ubiquitylation suggests that the H2B modification is a direct consequence of the recruitment of Rad6 to the *GAL1* promoter. Both the Gal4 activator and the Rad6-associated E3 ligase, Bre1, are required for Rad6 to bind to the *GAL1* promoter, suggesting that Gal4 might directly recruit the Rad6–Bre1 complex. Because we have previously shown that H2B ubiquitylation plays a role in the activation of *GAL1* transcription (Henry et al. 2003), the data indicate that Rad6 functions as a transcriptional coactivator in addition to its role in mediating postreplication DNA repair. H2B ubiquitylation was also found to increase transiently at the *PHO5* core promoter upon gene activation, suggesting that the dynamic regulation of this H2B modification could play a general role in the initiation of transcription. Consistent with the idea that H2B ubiquitylation is associated with transcriptionally active chromatin, the modified form of H2B was present at the promoters of both induced and constitutive genes but absent from regions of silent chromatin (Fig. 6A).

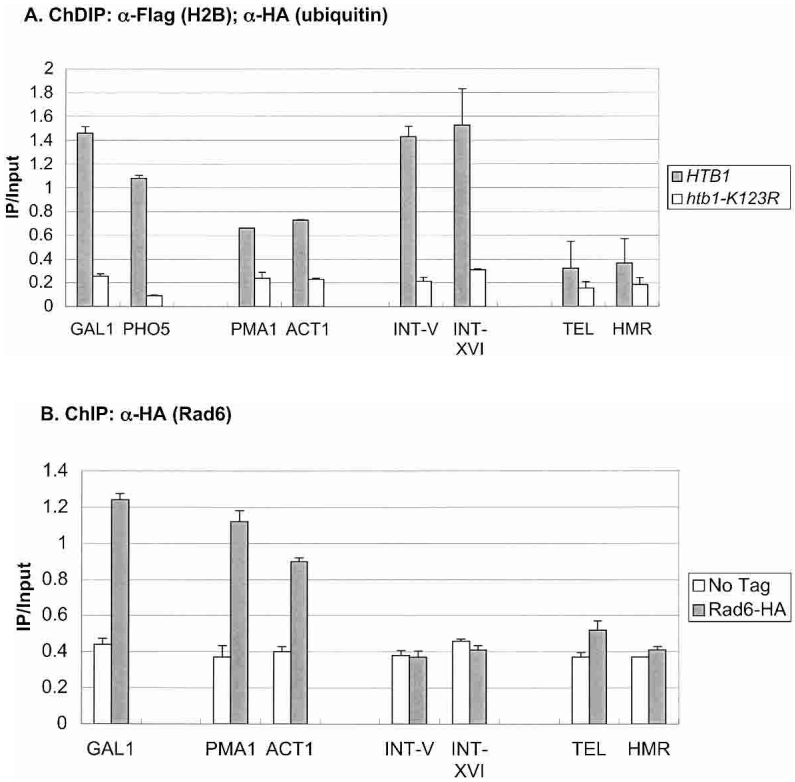

Figure 5. Distribution of ubiquitylated H2B. (*A*) ChDIP was sequentially performed with anti-Flag and anti-HA antibodies in strains YKH045 (*Flag-HTB1*) and YKH046 (*Flag-htb1-K123R*) after growth in YPD medium. PCR in real time was used to measure the level of H2B ubiquitylation at two transcriptionally silenced regions (*TELVI-R* and *HMRa*), two ORF-free intergenic regions (*INT-V* and *INT-XVI*), the core promoters of two highly transcribed constitutive genes (*PMA1* and *ACT1*), and the core promoters of the *GAL1* and *PHO5* genes after induction with galactose or no phosphate, respectively. (*B*) ChIP was performed with anti-HA antibodies in strains YKH010 (Rad6-HA *HTB1*) and JR5-2A (No tag *HTB1*) grown in YPD or induced with galactose, and PCR in real time was used to measure the level of Rad6-HA at the genomic regions described in panel *A*.

Rad6 and SAGA are sequentially recruited to the GAL1 promoter

Previous studies have shown that galactose induction is accompanied by the sequential appearance of proteins at the *GAL1–GAL10* promoter, with the SAGA complex being the first factor to bind after the Gal4 activator (Bhaumik and Green 2001; Larschan and Winston 2001; Bryant and Ptashne 2003). In this study, we have identified Rad6 as a new protein that appears at the *GAL1* promoter during galactose activation. Using ChIP, we found that the binding of Rad6 appears to temporally precede that of SAGA (Fig. 3C), suggesting that Rad6 may, in fact, be the earliest factor to associate with the *GAL1* promoter. Moreover, the recruitment of SAGA also appears to be independent of Rad6's activity in H2B ubiquitylation because Gcn5-HA associates with the *GAL1* promoter in both an *htb1-K123R* and *bre1*Δ mutant (Fig. 3B; data not shown). Thus, it is likely that Rad6 and SAGA are independently recruited to the *GAL1* UAS, with Rad6 binding first. Both factors bind to the *GAL* promoter only if the activator Gal4 is present (Fig. 2A; Bhaumik and Green 2001; Larschan and Winston 2001; Bryant and Ptashne 2003). SAGA interacts with Gal4 in vitro (Bhaumik and Green 2001), but we do not know whether Rad6 contacts Gal4 directly, or whether Rad6 and SAGA might contact the same surface of the activator. Bre1 is also required for Rad6 association with the *GAL1* UAS (Fig. 2B), and we therefore assume that a Rad6–Bre1 complex is recruited to the *GAL1* promoter. Thus, Bre1 could also provide a binding surface for Gal4. Although Gal4 is required to bring Rad6 to the promoter, we do not know how Rad6 is displaced. In the absence of H2B ubiquitylation, Rad6 is still recruited to the *GAL* UAS, but its turnover is slightly delayed (Fig. 1C). Perhaps the ubiquitylated template itself plays a role in releasing Rad6 from the *GAL* promoter. Alternatively, the recruitment of SAGA might contribute to the displacement of Rad6.

Rad6 not only appears to precede SAGA at the *GAL1* UAS, but it also binds transiently whereas SAGA binds stably. What is the significance of the different binding patterns between the two factors? We propose that the temporal difference in promoter association, which results in the staged recruitment of Rad6 and SAGA, provides a mechanism, first, to ubiquitylate H2B through Rad6-Bre1 and, subsequently, to deubiquitylate H2B through the Ubp8 subunit of SAGA (Henry et al. 2003). This would restrict both the period of H2B ubiquitylation as well as the level of the modified histone at the *GAL1* promoter, two conditions that we have previously shown to be important for the optimal transcription of *GAL1* (Henry et al. 2003). The transience of Rad6 promoter association might also contribute to the limited period and level of H2B ubiquitylation. SAGA, on the other hand, provides another function at the *GAL1* core promoter besides deubiquitylating H2B, which might account for its more stable association with the UAS. The Spt3 subunit of SAGA is required for the recruitment of TBP, and the continued presence of SAGA at the UAS could help to "tether" TBP to the core promoter during successive rounds of transcription initiation (Larschan and Winston 2001; Bhaumik and Green 2002).

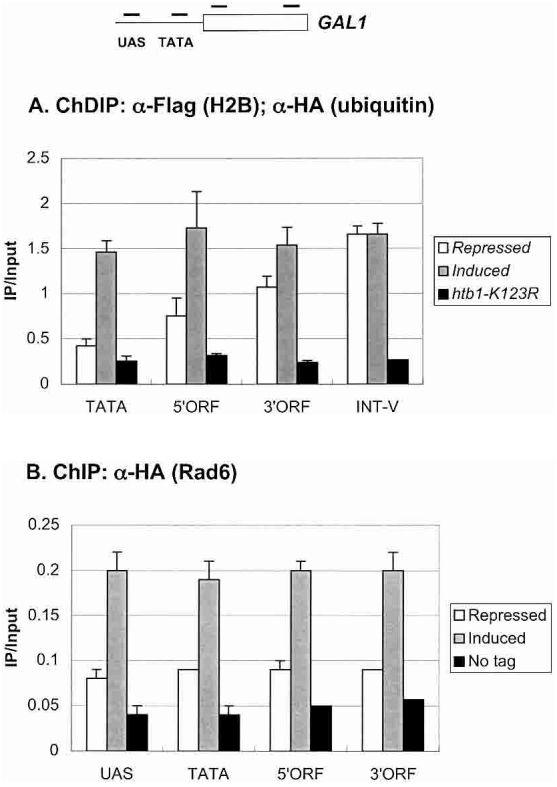

Figure 6. Ubiquitylated H2B and Rad6 are associated with the *GAL1* promoter and ORF. (*A*) ChDIP was performed in the *Flag-HTB1* strain YKH045 during growth in YPD medium (repressed, white bars) and after galactose induction (induced, gray bars). ChDIP was also performed in the Flag-*htb1-K123R* strain YKH046 after galactose induction (*htb1-K123R*, dark gray bars). DNA was analyzed at the *GAL1* TATA, 5′ ORF, and 3′ ORF regions by PCR in real time. The *INT-V* sequences served as a control for media differences. (*B*) ChIP was performed in the Rad6-HA strain YKH010 during growth in YPD medium (repressed, white bars) and after galactose induction (induced, gray bars). ChIP was also performed in the untagged control strain JR5-2A after galactose induction (No tag, dark gray bar). DNA was analyzed by PCR in real time at the *GAL1* UAS and the *GAL1* regions described in panel *A*. The approximate location of primers used for PCR analysis is shown relative to *GAL1* promoter regulatory elements and ORF.

Rad6 functions either directly or indirectly as a transcriptional repressor in silent chromatin and at the repressible *ARG1* gene, and this role is directly related to its activity in H2B ubiquitylation (Huang et al. 1997; Dover et al. 2002; Ng et al. 2002; Sun and Allis 2002; Turner et al. 2002). In contrast, Rad6 functions as a coactivator during galactose induction, also through its H2B ubiquitylation activity. Unlike SAGA, which is an essential coactivator at *GAL1* (Bhaumik and Green 2001; Larschan and Winston 2001), Rad6 is dispensable for *GAL1* transcription (data not shown). Gcn5 itself is also not required for *GAL1* transcription, and *gcn5*Δ mutants appear phenotypically similar to *htb1-K123R* mutants with respect to their modest defect in *GAL1* transcription (Fig. 7; Bhaumik and Green 2001; Larschan and Winston 2001). However, the presence of ubiquitylated H2B becomes very important for *GAL1* expression when the Gcn5 subunit of SAGA is absent (Fig. 7B), suggesting that Rad6-directed ubiquitylation of H2B and Gcn5-mediated acetylation of H3 play overlapping roles during galactose induction. Consistent with this view, *GAL1* transcript levels are significantly lower in an *htb1-K123R hht1-K14A* double mutant compared to each single mutant (C. Hillyer, unpubl. data). We do not know

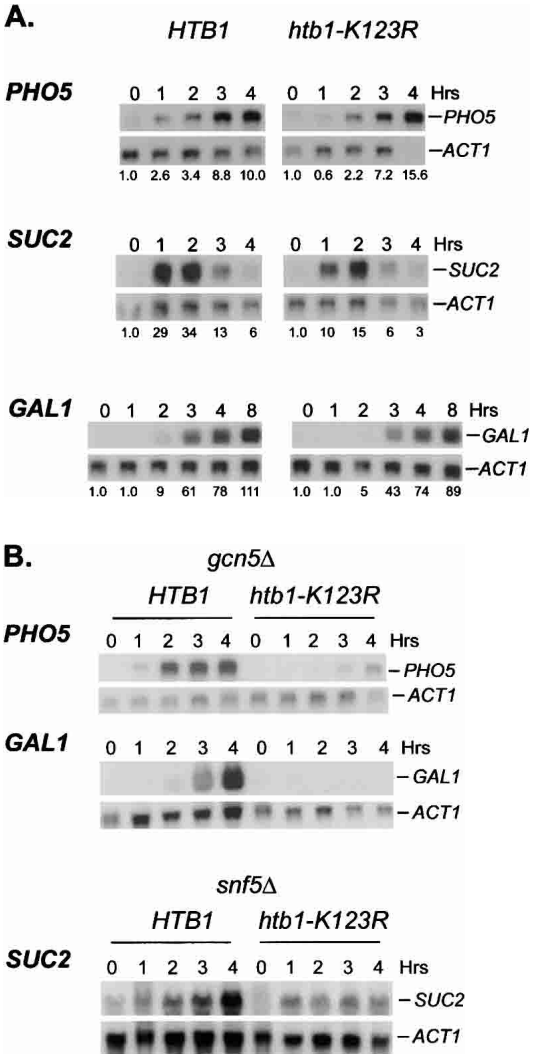

Figure 7. H2B ubiquitylation has overlapping functions with chromatin remodeling factors. (*A*) Cells from strain JR5-2A that contained either wild-type *HTB1* or the *htb1-K123R* allele were shifted from YPD medium to: no phosphate synthetic medium (*PHO5*), YP medium + 2% galactose (*GAL1*), or YP medium + 0.05% glucose (*SUC2*) for the indicated times. Northern blot analysis was performed with the indicated probes. The numbers beneath each lane represent transcript levels normalized to *ACT1* mRNA. (*B*) Northern blot analysis was performed in *gcn5*Δ (JR7-2B) or *snf5*Δ (JR16-6A) mutants that contained either wild-type *HTB1* or the *htb1-K123R* allele after cells were shifted to the media described in panel *A*. The *SUC2* blot was overexposed to show the effect of the *htb1-K123R* mutation on residual *SUC2* mRNA accumulation in the *snf5*Δ mutant.

what step in the galactose induction pathway is sensitive to the combined loss of H2B ubiquitylation and Gcn5. The Gal4 activator binds robustly to the *GAL* UAS elements in an *htb1-K123R gcn5*Δ double mutant upon galactose induction; however, we have found that the Swi/Snf complex, which is required for *GAL* transcription in some strain backgrounds, is not recruited to the *GAL1* promoter when both factors are absent (C. Hillyer, unpubl. data).

H2B ubiquitylation is a mark of active chromatin

H2B ubiquitylation is not restricted to the *GAL1* promoter but is found at other actively transcribed genes as well, including the inducible *PHO5* gene (Fig. 4). H2B is also transiently ubiquitylated at the *PHO5* promoter during gene induction, and, as seen for *GAL1*, the period of H2B ubiquitylation precedes the maximal accumulation of *PHO5* transcripts. This is consistent with the view that the H2B modification might play a general role during an early stage of gene activation. We assume, although we have not yet tested, that Rad6 ubiquitylates H2B at the *PHO5* promoter when phosphate is depleted. SAGA is recruited to this promoter by the Pho4 activator, and thus the Ubp8 subunit of SAGA might also regulate the deubiquitylation of H2B during *PHO5* activation (Barbaric et al. 2003). However, it was recently reported that SAGA-dependent hyperacetylation of nucleosomes at the *PHO5* promoter leads to histone eviction, and this mechanism could also account for the loss of ubiquitylated H2B (Reinke and Horz 2003). Ubiquitylated H2B is also present at the promoters of the constitutively transcribed *PMA1* and *ACT1* genes, and Rad6 is associated with the same promoters (Fig. 6). If transient ubiquitylation of H2B plays a general role in the initiation of transcription, it is predicted that the ubiquitin moiety will be continuously turned over at constitutive as well as inducible genes. Although neither the *PMA1* nor *ACT1* gene is regulated by the SAGA complex, other Ubps also target H2B for deubiquitylation (C.F. Kao, unpubl. data), and these Ubps might play a role in removing the ubiquitin moiety at SAGA-independent genes.

Ubiquitylated H2B appears to be excluded from regions of silenced chromatin, just like the H3 Lys 4 and Lys 79 methylation marks that it controls (Bernstein et al. 2002; van Leeuwen et al. 2002; Ng et al. 2003a). Hypomethylation of the H3 N termini is a prerequisite for Sir protein binding to silenced chromatin, and Sir binding to hypomethylated H3 N tails is postulated to block the Set1 and Dot1 HMTases from accessing their substrates (van Leeuwen and Gottschling 2002; van Leeuwen et al. 2002; Ng et al. 2003a). Rad6 is also excluded from silent chromatin, but Sir proteins are not known to interact with the H2B C tail. Thus, it could be that the structure of silenced chromatin generally inhibits Rad6 access to these regions, thereby blocking H2B ubiquitylation. Regardless, the combined data support the view that H2B ubiquitylation defines domains of active chromatin, either directly or through its role in the *trans*-histone regulation of histone H3 Lys 4 and Lys 79 methylation.

Materials and methods

S. cerevisiae *strains, plasmids, and growth conditions*

Yeast strains are all derivatives of W303-1A and are listed in Table 1. The genomic copies of the *RAD6* and *GCN5* genes were tagged with the triple HA epitope at their C termini using *his5* as a marker and sequences flanking the stop codons to direct integration at the appropriate chromosomal location (Longtine et al. 1998). Integrations were confirmed by PCR analysis and by Western blot analysis with anti-HA antibodies. Gene knockouts were performed by transformation with PCR products that contained the *URA3* gene flanked by 50–70 bp of DNA surrounding the ATG and stop codon of the appropriate gene. All gene knockouts were confirmed by PCR analysis and phenotypic screens. Plasmids carrying Flag-tagged *HTB1* or *htb1-K123R* genes have been previously described (Recht and Osley 1999; Robzyk et al. 2000). An *HA-UBI4* gene driven by the constitutive *GAPDH* promoter was carried on plasmid pRG145, which was kindly provided by R. Gardner and D. Gottschling (Fred Hutchinson Cancer Research Center). This gene was integrated at the *ura3-1* locus in strains carrying *Flag-HTB1* or *Flag-htb1-K123R* after digestion with StuI, following standard genetic procedures. Expression was confirmed by Western blot analysis with anti-Flag and anti-HA antibodies.

Cells were grown at 30°C in YPD medium or in SD medium supplemented with appropriate amino acids and bases. For gene induction experiments, cells were washed two times with sterile water and shifted into medium containing 2% galactose for *GAL1* induction, into medium containing 0.05% glucose for *SUC2* induction, and into no phosphate synthetic medium for *PHO5* induction (Vogel et al. 1989). For analysis of Rad6-HA and Gcn5-HA *GAL1* UAS association at short times after a shift to galactose-inducing conditions, cells were pregrown in YPD, washed, and resuspended in YP + 2% raffinose for 2 h, at which point galactose was added to a final concentration of 2%.

RNA analysis

Northern blot analysis was performed with 10–20 µg of total yeast RNA extracted from cells just before (0′) and at 1-h intervals after the shift to the appropriate medium for gene induction. Hybridization probes corresponding to the *GAL1*, *SUC2*, or *PHO5* ORFs were labeled by the method of random priming. Hybridization intensities were quantitated with a Molecular Dynamics PhosphorImager.

Chromatin immunoprecipitation

Yeast strains were grown in YPD medium to an $O.D._{600}$ of 0.5–0.7 and then shifted to YP medium containing 2% galactose. Immediately before and at 30-min intervals up to 120 min after the shift, 50 mL of cells were removed and fixed with 1% formaldehyde at room temperature for 15 min for analysis of histone modifications and for 30 min for analysis of Rad6-HA and Gcn5-HA binding. Fixation was stopped by the addition of glycine to 125 mM for 5 min, the cells were collected by centrifugation and washed two times with ice-cold TBS (100 mM Tris at pH 7.5, 0.9% NaCl). After the cell pellets were quick-frozen in EtOH-dry ice, they were stored at –80°C. The cell pellets were thawed on ice, resuspended in 500 µL of FA lysis buffer (50 mM HEPES-KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton

Table 1. S. cerevisiae *strains*

Strain	Genotype	Source
JR5-2A	*MATa htb-1-1 htb2-1 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100* <pRS314-*HTB1* or pRS314-*htb1-K123R*>	Robzyk et al. 2000
JR7-2B	*MATa htb1-1 htb2-1 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100 gen5*Δ*::TRP1* <pRS413-*HTB1* or pRS413-*htb1-K123R*>	Recht and Osley 1999
JR6-16A	*MATa htb1-1 htb2-1 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100 snf5*Δ <*pRS314-HTB1* or pRS314-*htb1-K123R*>	Recht and Osley 1999
YKH010	*MATa htb1-1 htb2-1 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100 RAD6-HA::his5* <pRS314-*HTB1*>	This study
YKH017	*MATa htb1-1 htb2-1 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100 RAD6-HA::his5* <pRS314-*htb1-K123R*>	This study
YKH012	*MATa htb1-1 htb2-1 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100 GCN5-HA::his5* <pRS314-*HTB1*>	Henry et al. 2003
YKH019	*MATa htb1-1 htb2-1 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100 GCN5-HA::his5* <pRS314-*htb1-K123R*>	This study
YKH045	*MATa htb1-1 htb2-1 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100 GAPDH::HA-UB14::URA3* <pRS314-*Flag-HTB1*>	Henry et al. 2003
YKH046	*MATa htb1-1 htb2-1 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100 GAPDH::HA-UB14::URA3* <pRS314-*Flag-htb1-K123R*>	Henry et al. 2003
Y131	*MATa hta1-htb1*Δ*::LEU2 hta2-htb2*Δ *leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can 1-100* <pRS426-HTA1-HTB1>	Robzyk et al. 2000
Y133	*MATa hta1-htb1*Δ*::LEU2 hta2-htb2*Δ *leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100* <*pRS426-HTA1-htb1-K123R*>	Robzyk et al. 2000
YCH001	*MATa htb1-1 htb2-1 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100 RAD6-HA::his5 gal4*Δ*::URA3* <pRS314-*HTB1*>	This study
YCH002	*MATa htb1-1 htb2-1 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100 RAD6-HA::his5 bre1*Δ*::URA3* <pRS314-*HTB1*>	This study
YCH003	*MATa htb1-1 htb2-1 leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100 RAD6-HA::his5 gen5*Δ*::URA3* <pRS314-*HTB1*>	This study

X-100, 0.1 sodium deoxycholate, 1 mM PMSF) supplemented with a fresh protease inhibitor cocktail (Sigma), and lysed by vortexing with glass beads for 15 min at 4° C. The cell lysates were sonicated six times for 10 sec each on a Branson Sonifier 250 set at 30% output and 100% duty cycle, with 30 sec cooling between each sonication cycle. The average size of the DNA fragments produced was between 300 and 500 bp. Following centrifugation at 15K for 30 min at 4° C in a microfuge, the solubilized chromatin was transferred to a fresh microfuge tube, and the volume was adjusted to 1 mL with FA lysis buffer. For analysis of input chromatin, 50–100 µL were removed.

For analysis of Rad6-HA and Gcn5-HA chromatin immunoprecipitations, 2–5 µl of anti-HA antibody (12CA5; Roche) were added to ~20 O.D. equivalents of solubilized chromatin and incubation was continued overnight at 4°C. Immune precipitates were collected by a 2-h incubation at 4°C with 40 µL of protein G sepharose beads (Amersham) equilibrated with FA lysis buffer and protease inhibitors. The beads were washed sequentially with 1.4 mL FA-lysis buffer, 1.4 mL FA-lysis buffer + 0.5 M NaCl, 1.4 mL LiCl solution (10 mM Tris at pH 8, 250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA), and 1.4 mL TE (pH 8) for 5 min each at 4°C, and the immune complexes were eluted twice at room temperature with 250 µL of 1% SDS, 0.1 M $NaCO_3$.

Chromatin double immunoprecipitation (ChDIP) to detect ubiquitylated H2B was carried out in strains YKH045 (*Flag-HTB1*; *HA-UBI4*) and YKH046 (*Flag-htb1-K123R*; *HA-UBI4*) by the method outlined in Henry et al. (2003), with the following modifications. One hundred microliters of M2 agarose beads (Sigma) equilibrated in FA lysis buffer were incubated with 40 O.D. equivalents of solubilized chromatin overnight at 4°C, the beads were collected by centrifugation and washed four times with FA lysis buffer, and the immune complexes were eluted with 500 µL FA lysis buffer containing 200 µg/mL 3× Flag peptide (Sigma) overnight at 4°C. One-tenth of the eluate was reserved for "input", and anti-HA antibody (12C5A, Roche) was added to a final concentration of 15 µg/mL to the remaining eluate, followed by incubation at 4°C overnight. The immune complexes were collected by incubation with 80 µL protein G sepharose beads (Amersham) for 3–5 h at 4°C and the beads were washed as described above. The beads were resuspended in 100 µL TE (pH 8) with 20 µg RnaseA (Sigma) and incubated at 37°C for 30 min. Following a wash with 1 mL TE, the immune complexes were eluted from the beads by sequential incubation in 500 µL of 1% SDS/50 mM Tris (pH 8) for 5 min at 100°C and for 10 min at 65°C. After adjusting the NaCl concentration in each sample to 0.2 M, the cross-links were reversed by incubation at 65°C overnight. DNA was extracted according to published procedures (Hecht et al. 1996; Kuo and Allis 1999) and resuspended in 100 µL TE ("input") or 25 µl TE ("IP").

PCR analysis

Quantitative PCR in real time was performed using a SYBR Green master mix and an ABI 7000 Prism Sequence Detection system, both from Applied Biosystems. Triplicate samples containing 5 µL of 1:20–1:100 diluted IP DNA and 1:500–1:1000 diluted input DNA were analyzed, and all reactions were performed in the linear range of amplification. PCR primers were designed using the software program Primer Express (ABI). An intergenic sequence on chromosome V (*INT-V*) that is not regulated by carbon source or low phosphate served as an internal control. Conventional PCR was carried out with 5 µL of IP DNA

and typically 5 µL of 1:50 diluted input DNA, using a MJ Research Thermocycler. PCR products were separated on a 10% polyacrylamide-SDS gel, which was stained with ethidium bromide and photographed on a Nucleotech gel documentation system.

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