Cell cycle-regulated histone acetylation required for expression of the yeast *HO* gene

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Expression of the yeast *HO* gene in late G_1 of the cell cycle requires the SWI/SNF chromatin remodeling complex, the Gcn5p histone acetyltransferase, and two different sequence-specific transcriptional activators, Swi5p and Swi4p/Swi6p. We have used chromatin immunoprecipitation assays to investigate the role of each of these *trans*-acting factors in establishing a cell cycle-regulated domain of histone acetylation surrounding the *HO* upstream regulatory region. We detect a ~1-kb domain of H3 and H4 acetylation that is established in mid-G_1, prior to and independent of *HO* transcription, which then declines with kinetics similar to inactivation of *HO*. This cell cycle burst of histone acetylation requires Gcn5p, SWI/SNF, and the Swi5p activator, but occurs in the absence of the Swi4p activator. We also find that inactivation of the Sin3p/Rpd3p deacetylase complex leads to a high level of acetylation at the *HO* locus throughout the cell cycle. We propose a sequential model for activation of *HO* in which the Swi5p-dependent recruitment of the Gcn5p acetyltransferase requires chromatin remodeling events by the SWI/SNF complex.

[*Key Words*: Cell cycle; yeast; *HO* gene; histone acetylation; chromatin remodeling]

Received March 30, 1999; revised version accepted April 13, 1999.

Transcriptional activation in eukaryotes requires that transcription factors gain access to their target genes in a chromatin environment. The wrapping of DNA around histone proteins to form nucleosomes, as well as the higher-order folding of nucleosomal arrays, produce structures that are inaccessible to the transcription machinery. Naturally, eukaryotes have evolved mechanisms with which to contend with these repressive chromatin structures (Workman and Kingston 1998).

In recent years a large number of protein complexes capable of modifying chromatin structure have been identified. These chromatin remodelers fall into two general classes: the ATP-dependent remodelers and the histone acetyltransferases (HATs). The prototype of the ATP-dependent class of remodeling enzymes is the SWI/SNF complex of *Saccharomyces cerevisiae*, which is required for the transcriptional activation of a subset of genes and for the full functioning of several transcriptional activators (Laurent and Carlson 1992; Laurent et al. 1993; Peterson et al. 1994; Peterson 1996; Burns and Peterson 1997; Ryan et al. 1998). In vitro, SWI/SNF uses the energy of ATP hydrolysis to alter DNA-histone contacts to make nucleosomal DNA more accessible to transcription factors (Cote et al. 1994, 1998; Imbalzano et al. 1994, 1998; Utley et al. 1996) and restriction enzymes (Logie and Peterson 1997). Yeast have also provided the first example of a transcription-related HAT, Gcn5 (Brownell et al. 1996), which is a member of several multisubunit complexes (Grant et al. 1997; Pollard and Peterson 1997; Saleh et al. 1997; Eberharter et al. 1998). *GCN5*-dependent HAT complexes acetylate nucleosomal histones and can promote transcription from nucleosomal templates in vitro (Steger et al. 1998; Utley et al. 1998; Ikeda et al. 1999). In vivo, *GCN5* is required for expression of a set of genes that overlaps with those that require SWI/SNF; in fact, *gcn5 swi/snf* double mutants exhibit synthetic phenotypes (Pollard and Peterson 1997; Roberts and Winston 1997). Residues in GCN5p that are critical for HAT activity in vitro are also required for Gcn5p's transcriptional activation function in vivo (Candau et al. 1997; Wang et al. 1998), and *GCN5* has been shown to be required for promoter-directed acetylation at the *HIS3* gene in vivo (Kuo et al. 1998). Although Gcn5p-containing HAT complexes have been shown to interact with several activator proteins in vitro (Utley et al. 1998), little is known about how these chromain remodelers are targeted to their sites of action in vivo.

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One of the yeast genes whose expression requires both Gcn5p and SWI/SNF is *HO* (Pollard and Peterson 1997; Perez-Martin and Johnson 1998), which encodes a site-specific endonuclease that initiates the process of mating-type interconversion. *HO* expression is tightly regulated by cell type and during the cell cycle, being expressed in only a small window in late G_1 and only in haploid mother cells (cells which have budded previously) (for review, see Herskowitz et al. 1992; Nasmyth 1993). This complex pattern of *HO* expression requires an unusually large upstream regulatory region (~2000 bp), and a host of positive and negative *trans*-acting factors, including SWI/SNF, Gcn5p, the sequence-specific activators Swi5p and Swi4p/Swi6p, the Sin3p/Rpd3p deacetylase complex, and the daughter-specific repressor, Ash1p (Breeden and Nasmyth 1987; Sternberg et al. 1987; Herskowitz et al. 1992; Nasmyth 1993; Jansen et al. 1996; Sil and Herskowitz 1996). Swi5p is involved in mother/daughter regulation and binds to two sequence elements located 1300- and 1800-bp upstream from the *HO* mRNA start site. The *SWI4* and *SWI6* genes encode two subunits of a transcriptional activator, called SBF, that binds in late G_1 to 10 cell cycle boxes located within a 900-bp region upstream of the *HO* mRNA start site. Binding of SBF in late G_1 is not sufficient for activation of *HO*, as a subsequent step requires phosphorylation by a Cdc28p/Clnp kinase at the G_1/S boundary (Start) (Harrington and Andrews 1996; Koch et al. 1996). The inactivation of *HO* expression after Start requires the activity of an S-phase Cdc28p/Clbp kinase (Amon et al. 1993).

In this study, we have examined the histone acetylation pattern of the *HO* promoter during G1 and early S phase. By taking advantage of mutations in the various regulators of *HO*, we have determined the activator and remodeler dependence of the changes in histone acetylation. Our results show that there is a cell cycle-regulated wave of *GCN5*-dependent histone acetylation that is restricted to ~1 kb of the *HO* upstream regulatory region encompassing the 10 cell cycle boxes and the TATA element. *GCN5*-dependent acetylation requires Swi5p and SWI/SNF, but occurs in the absence of Swi4p and precedes transcription. In contrast, inactivation of the Sin3p/Rpd3p deacetylase complex leads to an expanded domain of acetylation as well as high levels of histone acetylation throughout the cell cycle. We propose a sequential order of action for the regulators of *HO* expression in which the Swi5p activator recruits SWI/SNF whose remodeling activity then targets a Gcn5p-containing HAT complex to the promoter. Acetylation of nucleosomes that encompass the cell cycle boxes may then facilitate binding of the Swi4p/Swi6p complex, which acts very late in the activation cycle. And finally, the Sin3p/Rpd3p deacetylase complex may play a significant role in erasing acetylation events, ensuring that the *HO* locus has a low level of histone acetylation prior to the beginning of the next cell cycle. These data support the view that chromatin remodeling factors are recruited by site-specific activators, and suggest that two different classes of chromatin remodelers may function at separate and successive steps in gene activation.

Results

Nucleosomes encompassing the HO *promoter are dynamically acetylated in a cell cycle- and* GCN5*-dependent manner*

Expression of the *HO* gene is restricted to a short window in late G_1 of mother cells. *HO* expression is repressed in daughter cells by the repressor protein, Ash1p, and thus inactivation of *ASH1*, allows expression of *HO* in both mothers and daughters. Importantly, a deletion of the *ASH1* gene does not disrupt other modes of *HO* regulation, such as haploid specificity and cell cycle regulation. Therefore, we have used an isogenic set of strains for our analyses that harbor a deletion of *ASH1*, allowing us to obtain homogeneous cell populations in which *HO* is uniformly expressed in late G_1 of the cell cycle. To examine acetylation events throughout G_1 and early S phase, we synchronized cells in G_2/M with nocodazole, and then washed out the nocodazole to allow the cells to progress through a synchronous cell cycle. Cell aliquots were taken at 15-min intervals following release from the nocodazole block, and these samples were analyzed for position in the cell cycle (by budding index), *HO* expression (by primer extension of RNA), and histone acetylation [by chromatin immunoprecipitation (ChIP)]. Figure 1A shows a typical nocodazole arrest/release timecourse for a *GCN5 ash1* strain, showing budding index and *HO* expression. After 3 hr of incubation with nocodazole, ~100% of the cells are arrested in the G_2/M phase of the cell cycle as indicated by the large budded phenotype (time zero). Cells begin to enter G_1 (cells with no buds) between 30 and 45 min after release from the nocodazole block, and cells traverse the G_1/S boundary ~90 min after release (appearance of small buds). At 120 min after release, most cells have entered S phase as indicated by the high proportion of budded cells. As expected, *HO* transcripts are first detected by primer extension at 75–90 min after release, immediately before the onset of S phase at 90 min (Fig. 1A).

GCN5 ash1 and *gcn5 ash1* cells were synchronized with nocodazole, timepoints were fixed with formaldehyde, and chromatin was isolated and processed for immunoprecipitation as described in Materials and Methods. To investigate the pattern of histone acetylation at the *HO* promoter, fragmented chromatin was immunoprecipitated with antibodies specific to a diacetylated form of histone H3 acetylated at lysines 9 and 14 (αH3ac.9/14). These lysines are the preferred sites of acetylation by Gcn5p in vitro (Kuo et al. 1996), and a mutation in H3 that changes lysine 14 to arginine confers a strong, synthetic growth defect in *gcn5* cells (Zhang et al. 1998). Immunoprecipitated chromatin and the input material were deproteinized and applied to slot blots, which were then hybridized with either total genomic DNA, rDNA, or a DNA probe specific for the *HO* TATA region (see Fig. 2A). PhosphorImager scans of representative slot blots probed with the *HO* TATA probe are shown in Figure 1B. Quantitation of these blots as well as blots probed with genomic or rDNA is shown in Figure 1C. Quantitated data (Fig. 1C) are presented as IP

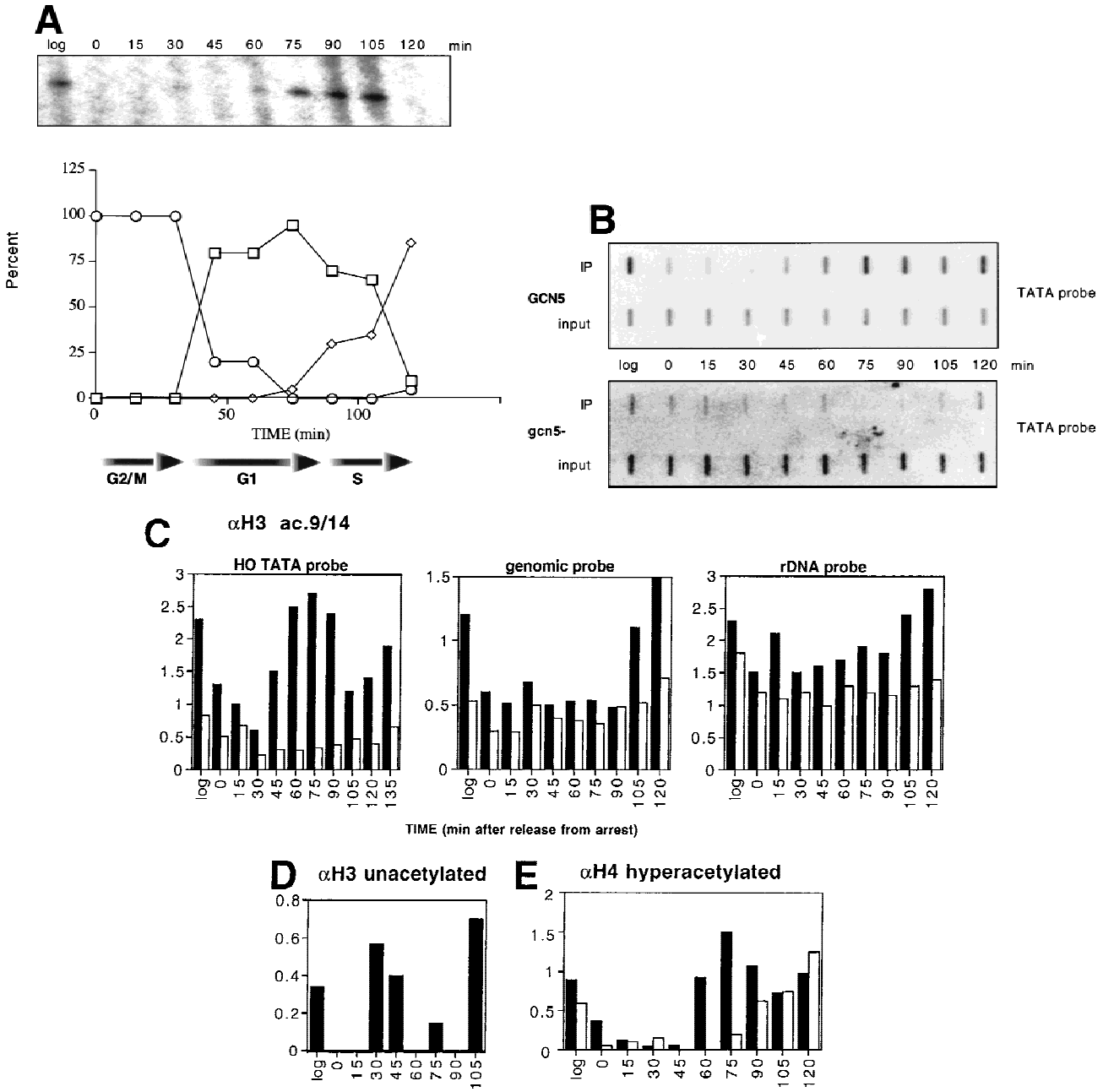

Figure 1. Nucleosomes at the TATA region of *HO* are subject to *GCN5*-dependent, transient acetylation prior to transcription. (*A*) Budding index and primer extension analysis of *HO* transcripts show the cell cycle-dependence of *HO* expression. Cells were arrested in G_2/M by treatment with nocodazole, as described in Materials and Methods. Percentages of cells with no buds (□) (G_1), small buds (◇) (S phase), or large buds (○) (G_2/M) were determined at the times shown after release from nocodazole arrest. Samples were taken from these timepoints for either RNA analysis or for ChIPs (see below). *HO* transcripts are detectable by primer extension in late G_1 and peak at the onset of S phase (appearance of small buds; 90–105 min in wild-type cells). (*B*) Results of ChIP assays for either wild-type or *gcn5*- cells. Chromatin obtained from cells released from nocodazole arrest as described above was immunoprecipitated with antibodies that recognize histone H3 acetylated at positions 9 and 14 (αH3ac.9/14). DNA obtained from either input or immunoprecipitated material was applied to slot blots. Representative slot blots probed for the *HO* TATA region are shown. (*C*) Quantitation of representative slot blots. Blots were quantitated with a PhosphorImager; the results are expressed as the ratio of the bound to the input material (IP efficiency). The graph at *left* shows the results for the *HO* TATA probe for either *GCN5*$^+$ or *gcn5*$^-$ cells, showing the *GCN5*-dependent peak of acetylation between 60 and 90 min after release from nocodazole arrest. The high levels of acetylation detected in log phase cells, as well as in early S phase, are also partially *GCN5* dependent. The *middle* graph depicts the global levels of *GCN5*-dependent acetylation using the genomic DNA probe, and acetylation detected by the rDNA probe is shown at *right*. The log- and early S-phase levels of acetylation are high in both total DNA and rDNA, but the late-G_1 peak is specific to *HO*. Data shown are representative of four separate experiments with ChIP extracts from two independent cell synchronizations. (*D*) ChIP analysis of the wild-type samples from *C* using antibodies against unacetylated H3. The smaller scale on this graph reflects the weaker IP efficiency of these antibodies. (*E*) ChIP analysis of wild-type and *gcn5*- cells with antibodies recognizing hyperacetylated histone H4. (Solid bars) Gcn5p; (open bars) gcn5.

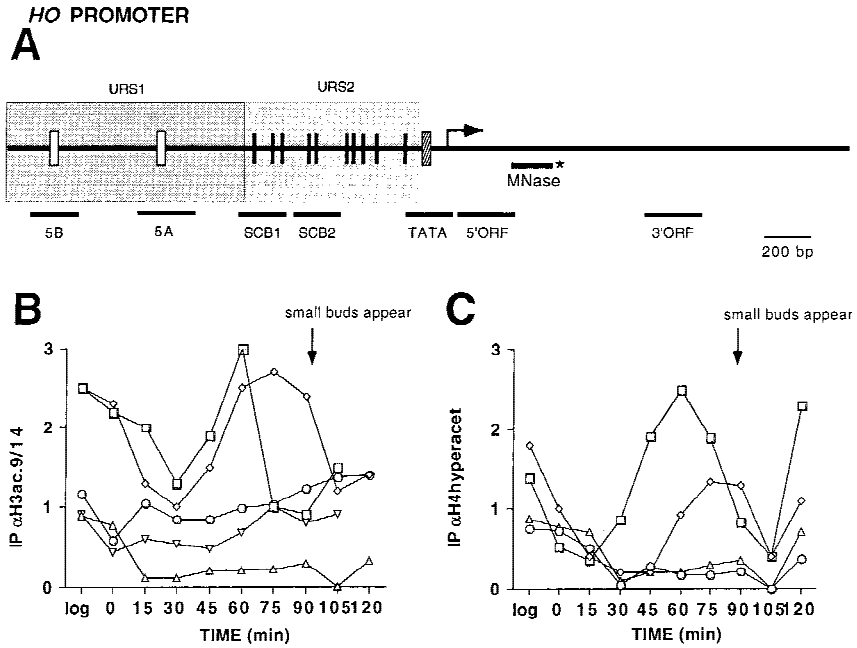

Figure 2. Acetylation at the *HO* promoter is limited to a 1-kb region encompassing the TATA box and the Swi4p/Swi6p-binding sites. (*A*) Schematic of the *HO* promoter showing the probes used in this study. (Hatched bars) TATA; (solid bars) SWI cell cycle box (SCB); (open bars) Swi5p binding site. (*B*) Results of probe scan of *HO* for samples precipitated with αH3ac.9/14. The slot blots shown in Fig. 1B were stripped and hybridized with a variety of DNA probes covering the *HO* promoter and coding region. The results for the TATA probe are the same as shown in Fig. 1C. Probes for the Swi4p/Swi6p-binding sites (SCB1 and SCB2) also detect a peak of acetylation that occurs slightly before the peak detected at the TATA. Probes for either of the Swi5p-binding sites (5A and 5B) or for the *HO* coding region (5′ ORF or 3′ ORF) do not detect a peak of acetylation. (*C*) Results of probe scan of *HO* for samples precipitated with antibodies to hyperacetylated H4. (*B*,*C*) (△) 5′ ORF; (◇) TATA; (□) SCB1; (○) 5B; (▽) 5A.

efficiencies, in which the signal obtained from the immunoprecipitated material is normalized to the input sample. In the *GCN5 ash1* cells, there is a strong, transient peak of H3 acetylation that begins 45 min after release from the nocodazole block and declines between 90 and 105 min (Fig. 1C, left). This peak of acetylation precedes *HO* transcription by ~15–30 min (Fig. 1A) and declines with kinetics similar to the disappearance of *HO* transcripts (Fig. 1A). Furthermore, G_1 acetylation of the *HO* locus is absent in *gcn5 ash1* cells (open bars), and it does not occur at the rDNA locus (right) or throughout the yeast genome (center). Thus, histone H3 that is assembled into nucleosomes that encompass the *HO* TATA element is dynamically acetylated in early G_1 by a *GCN5*-dependent acetyltransferase.

Gcn5p can also acetylate histone H4 in vitro (Kuo et al. 1996), and substitution of lysines within the H4 amino-terminal domain also show synthetic phenotypes with *gcn5* mutants in vivo (Zhang et al. 1998). To investigate histone H4 acetylation at the *HO* locus during G_1 and early S phase, we immunoprecipitated fixed chromatin samples with antibodies that recognize hyperacetylated histone H4, and immunoprecipitated material was detected with the *HO* TATA probe. As shown in Figure 1E, there is a transient peak of H4 acetylation that coincides with the peak observed for H3 (60–90 min postrelease). Furthermore, this peak of H4 hyperacetylation is also *GCN5* dependent (open bars).

If *HO* chromatin is dynamically acetylated during G_1, then we should expect that the level of unacetylated histone H3 would show a reverse pattern, declining in G_1. To test this possibility, we immunoprecipitated a subset of the same chromatin samples used in Figure 1C using antibodies directed against unacetylated histone H3 (Fig. 1D). As predicted, and in contrast to our results obtained with antibodies to acetylated histones, the level of unacetylated histone H3 at the *HO* locus is high in late anaphase/early G_1 (30 min), and decreases as cells enter (45 min) and traverse G_1 (75 min). Thus, the immunoprecipitation profile for unacetylated H3 does not show a G_1 peak, but is the reciprocal of that seen for acetylated histones.

GCN5 *contributes to genome-wide acetylation of histone H3 during S phase*

We also routinely observe an additional increase in H3 acetylation as cells enter S phase (Fig. 1C, 105–120 min). This second peak of H3 acetylation is most apparent with the genomic and rDNA probes, and it is partially dependent on *GCN5* (right and center, respectively). The hyperacetylation of histone H4 also increases dramatically in S phase, but in this case the increase does not depend on *GCN5* (Fig. 1E). A role for Gcn5p in S-phase H3 acetylation may also provide an explanation for the partial *GCN5* dependence of genome-wide acetylation observed in chromatin isolated from logarithmically growing cells (Fig. 1, bars marked log; see also Kuo et al. 1998). Thus, whereas the G_1 peak of H3 and H4 acetylation is specific for the *HO* locus, Gcn5p also appears to contribute to a global acetylation of histone H3 during S phase.

G_1*-specific acetylation at the* HO *locus is restricted to a 1-kb region of upstream regulatory sequences*

Previous studies have shown that *GCN5* is required for H3 acetylation surrounding the *HIS3* gene, and that

these acetylation events may be restricted to only ~200 bp of upstream sequences (i.e., one to two nucleosomes; Kuo et al. 1998; M.H. Kuo and C.D. Allis, unpubl.). We investigated the extent of H3 and H4 acetylation at the *HO* locus by using hybridization probes that span the entire *HO* locus (Fig. 2A). In addition to the TATA probe described above, we generated probes against each of the Swi5p-binding sites (Fig. 2A), against regions containing several of the Swi4p/Swi6p-binding sites (SCB1 and SCB2), as well as probes for the coding region of *HO* (5′ORF and 3′ORF). We also synthesized probes to the coding regions of two genes that surround the *HO* locus, *SSB1* and *GCS1* (not shown). Slot blots such as those shown in Figure 1 were then sequentially hybridized with these nine DNA probes. A subset of these results is shown in Figure 2B for H3 diacetylation and Figure 2C for H4 hyperacetylation.

The probe scan provided two important results. First, H3 and H4 acetylation at the *HO* locus during G_1 is restricted to ~1 kb of upstream regulatory sequences that encompasses the TATA box (TATA probe) and all of the Swi4p/Swi6p-binding sites (SCB1 and SCB2 probes) (Fig. 2B and data not shown). MNase digestion mapping indicates that this region contains at least six nucleosomes (J. Krebs and C.L. Peterson, unpubl.). The G_1 peak of acetylation is not observed either upstream of this region (Swi5p-binding sites A and B) or in the coding region of *HO* (ORF probes) (Fig. 2; data not shown). Acetylation is also low in the coding regions of genes that flank *HO* (data not shown). Second, we repeatedly observe acetylation at the distal Swi4p/Swi6p-binding sites (SCB probes) earlier in the cell cycle than that seen at the TATA region (peak at 60′ for SCB probes vs. peak at 75′ for TATA probe). This is consistent with an acetyltransferase being targeted to the far upstream region of the *HO* promoter, which then progresses downstream toward the TATA element, creating a wave of histone acetylation.

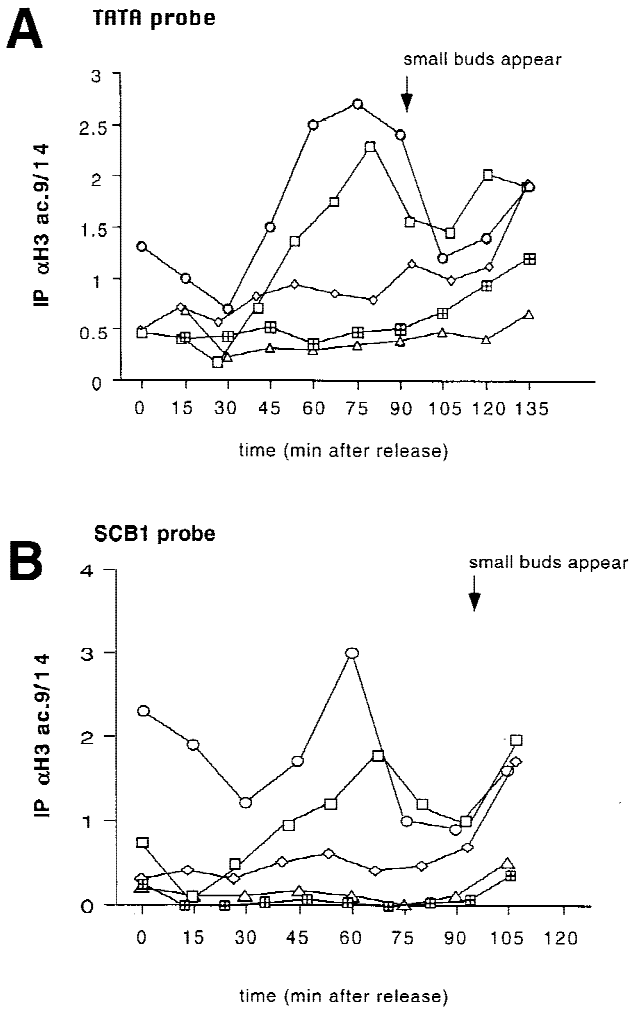

Figure 3. The cell cycle-regulated domain of histone H3 acetylation at *HO* requires Gcn5p, Swi5p, and an intact SWI/SNF complex. *SWI+*, *swi2–*, *swi5–*, or *swi4–* cells were synchronized with nocodazole and samples were fixed for chromatin IP after synchronous release. ChIPs were performed with the antibody to diacetylated histone H3 (ac.9/14). The *swi4* and *swi2* mutants showed a significant delay in recovering from nocodazole arrest and thus synchronies are aligned by the time of bud emergence (arrow at top). Time scale shown on the x axis is from the *SWI+* synchrony. Data shown are the result of four separate experiments from each strain with two independent cell synchronies. (*A*) Acetylation at the *HO* TATA region in wild-type (WT) (○) cells and in *swi5* (⊞), *swi2* (◇), *swi4* (□), and *gcn5* (△) mutants. (*B*) Acetylation over the SCB elements in wild-type cells and in *swi5*, *swi2*, *swi4*, and *gcn5* mutants.

Acetylation of histone H3 at the HO *promoter requires Swi5p and the SWI/SNF complex*

Next, we wished to determine whether the recruitment or activity of the *GCN5*-dependent HAT might require the Swi5p or Swi4p site-specific transcriptional activators or the chromatin remodeling activity of the SWI/SNF complex. We therefore constructed an isogenic set of *ash1*Δ strains that also carried a deletion of either *SWI2* (which encodes the ATPase subunit of SWI/SNF), *SWI5*, or *SWI4*. Synchronous populations of each of these strains were generated by nocodazole arrest and release, and histone H3 acetylation at the *HO* TATA region was analyzed by ChIP (Fig. 3). In a *swi5 ash1* double mutant, H3 acetylation at the *HO* locus is not established during G_1, although acetylation at *HO* and throughout the genome still increases at S phase (Fig. 3A and data not shown). Likewise, there is no significant peak of H3 acetylation in *swi2 ash1* cells, whereas overall acetylation in log and S phase shows wild-type levels (Fig. 3A; data not shown). *SWI5* and *SWI2* are also required for acetylation events outside the *HO* TATA box, as H3 acetylation over the SCB1 region also requires Swi5p and an intact SWI/SNF complex (Fig. 3B). Thus, recruitment or activity of a *GCN5*-dependent histone acetyltransferase requires one sequence-specific activator, Swi5p, and the SWI/SNF chromatin remodeling complex.

Regulated expression of *HO* relies on the combined

action of two distinct transcriptional activators, Swi5p and Swi4p/Swi6p. Whereas H3 acetylation requires the Swi5p activator, we observe a normal peak of H3 acetylation at both the *HO* TATA and the SCB1 regions in a *swi4 ash1* double mutant (Fig. 4A,B). Thus, these data indicate that the Swi4p/Swi6p activator may function at a step that is subsequent to the *GCN5*-dependent acetylation of the *HO*-upstream regulatory region. It is also important to note that there is no *HO* transcription in a *swi4* mutant (Stern et al. 1984; Breeden and Nasmyth 1987; J.E. Krebs, and C.L. Peterson, unpubl.) and we can therefore conclude that acetylation not only appears to precede transcription in wild-type cells, it can also occur in the complete absence of transcription.

Role of the Sin3p/Rpd3p deacetylase complex

Although the data presented in Figure 3 identifies the *trans*-acting factors required for establishing cell cycle-regulated acetylation at the *HO* locus, these results do not address how these acetylation events are eliminated when *HO* is repressed in early S phase or how the deacetylated state is established as cells enter G_1. For instance, we find that the levels of acetylated histones H3 and H4 decline precipitously in early S phase just prior to the inactivation of *HO* transcription (Figs. 1–3). Likewise, at the start of G_1, H3 acetylation at the *HO* locus is much lower than the levels observed throughout the genome (Figs. 1C and 2B). One possibility is that these deacetylation events might be controlled by the Sin3p/Rpd3p deacetylase complex (Kasten et al. 1997), because *SIN3* (*SDI1*) and *RPD3* (*SDI2*) were identified as negative regulators of *HO* expression (Nasmyth et al. 1987; Sternberg et al. 1987; Stillman et al. 1994). To address this possibility, we constructed a *sin3 ash1* double mutant, and a synchronous cell population was generated by nocodazole arrest and release. Histone H3 acetylation at the *HO* locus was then analyzed during the cell cycle by ChIP (Fig. 4). Whereas wild-type cells exhibit a dramatic burst of histone acetylation as cells enter G_1, inactivation of *SIN3* leads to a high level of H3 acetylation that shows little cell cycle periodicity (Fig. 4A). In particular, H3 acetylation does not decline at the G_1/S boundary, but rather the level of H3 acetylation increases further as cells enter S phase. Furthermore, H3 acetylation is also high in the *HO* coding region and upstream of the SCB elements (Fig. 4B,C). Although histone deacetylation in early S phase seems to parallel inactivation of *HO* expression in wild-type cells, constitutive histone acetylation does not alter the cell cycle timing of *HO* activation (J.E. Krebs and C.L. Petersen, data not shown; see also Nasmyth et al. 1987). Thus, inactivationof the Sin3p/Rpd3p deacetylase complex disrupts the size of the acetylated domain and eliminates the cell cycle-regulated acetylation of the *HO* upstream region. The cell cycle timing of *HO* expression, however, appears to be primarily controlled by the regulation of Swi4p/Swi6p by Cdc28p/cyclin kinases (Harrington and Andrews 1996; Koch et al. 1996).

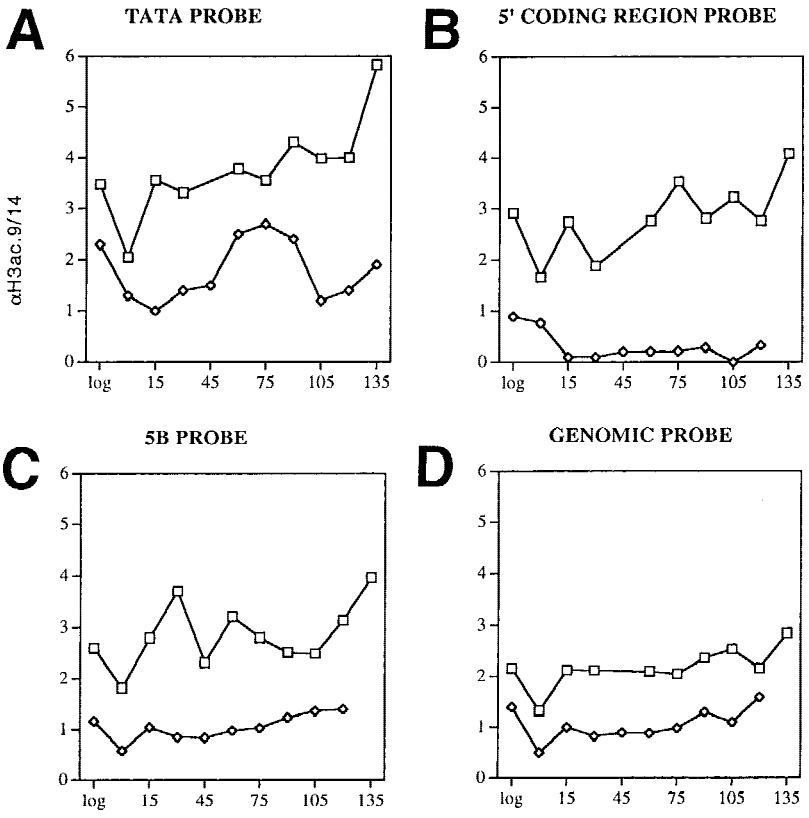

Figure 4. The entire *HO* gene is acetylated throughout the cell cycle in *sin3Δ* cells. *sin3*$^-$ cells were synchronized with nocodazole and samples were fixed for chromatin IP after synchronous release. ChIPs were performed with the antibody to diacetylated histone H3 (ac.9/14). In each panel, data from *sin3* cells (□) are compared with wild-type (◇). (*A*) Acetylation at the *HO* TATA region in wild-type cells and in *sin3* mutants. (*B*) Acetylation at the 5′ end of the *HO* coding region in wild-type cells and in *sin3* mutants. (*C*) Acetylation at the upstream Swi5p-binding site in wild-type cells and in *sin3* mutants. (*D*) Genomic H3 acetylation in wild-type and *sin3* cells.

Discussion

We have shown here that there is a cell cycle-regulated acetylation of histones H3 and H4 that occurs throughout a ~1000-bp region of the *HO* promoter. These acetylation events require the Gcn5p HAT, a site-specific activator, Swi5p, and the ATP-dependent chromatin remodeler, SWI/SNF. In contrast, cell cycle-regulated acetylation does not require the Swi4p/Swi6p activator and it precedes, and is independent of, transcription. Furthermore, inactivation of *SIN3* leads to high levels of histone acetylation at the *HO* locus throughout the cell cycle, suggesting that the Sin3p/Rpd3p complex may control deacetylation events at the *HO* promoter. Our studies are consistent with a sequential model for activation of *HO* transcription that involves at least two functionally dependent chromatin remodeling events.

The first step in the activation of *HO* transcription is likely to involve the Swi5p activator. Swi5p is expressed in S phase, in which it remains localized in the cytoplasm until it enters the nucleus in late anaphase after inactivation of a Clbp/Cdc28p kinase (Moll et al. 1991; Tebb et al. 1993). After its arrival in the nucleus, Swi5p is competent for DNA binding and transcriptional activation, because *SWI5* is required for expression of a number of genes in early G_1, such as *ASH1* (Bobola et al. 1996), *SIC1* (Knapp et al. 1996; Toyn et al. 1997), *EGT2* (Kovacech et al. 1996), *CDC6* (Piatti et al. 1995), *PCL2* and *PCL9* (Aerne et al. 1998). Although Swi5p probably binds in early G_1 to its two sites far upstream of the *HO* TATA element (see Fig. 2A), *HO* transcription does not ensue until much later at the G_1/S boundary. Swi5p may carry out its function prior to the actual activation of *HO* transcription, because Swi5p is rapidly degraded during G_1 (Tebb et al. 1993). Because Swi5p is required for *GCN5*-dependent acetylation of nucleosomes that encompass the downstream cell cycle boxes, this early function may involve the recruitment or activation of a Gcn5p-containing HAT complex.

Our data also indicate that SWI/SNF acts at an early step in *HO* expression, as it is required for the recruitment or activity of a *GCN5*-dependent acetyltransferase in mid-G_1. Although our acetylation data cannot define an order of action for Swi5p and SWI/SNF, Nasmyth and colleagues (Cosma et al. 1999) have used a ChIP assay to show that Swi5p associates with the *HO* locus prior to SWI/SNF, and that Swi5p is required for subsequent SWI/SNF recruitment. Once SWI/SNF is recruited by Swi5p, our data suggests that remodeling by SWI/SNF is required for the recruitment or activity of a *GCN5*-dependent HAT.

In mid-G_1, nucleosomes within a ~1-kb segment of the *HO* upstream regulatory region are acetylated by a *GCN5*-dependent HAT complex. We propose that this HAT complex is targeted to the promoter either directly by interaction with Swi5p, or as a result of SWI/SNF remodeling. Acetylation appears to initiate at the distal SCB elements and then continues into the TATA region, consistent with a movement of the acetyltransferase complex unidirectionally along the promoter. We propose that these acetylation events are required to facilitate the binding of Swi4p/Swi6p to its binding sites located within this domain. SWI/SNF may also be required for this step, because SWI/SNF is required for the UAS activity of this *SWI4*/*SWI6*-dependent UAS (Nasmyth 1987; Sternberg et al. 1987). Once Swi4p/Swi6p are bound to the *HO* upstream region, transcription can only be activated at the G_1/S boundary when a Cdc28p/Clnp kinase directly or indirectly stimulates Swi4p/Swi6p activity. We anticipate that this last step may involve the actual recruitment of the general transcription machinery or RNA polymerase II holoenzyme.

Role of histone deacetylases in the regulation of HO *expression*

Whereas Swi5p and SWI/SNF are required to establish a domain of histone acetylation in mid-G1, an intact Sin3p/Rpd3p deacetylase complex is required to erase these acetylation events in early S phase and to ensure that acetylation is low as cells enter the subsequent cell cycle. Consequently, in a *sin3* deletion mutant, the *HO* locus is encompassed by highly acetylated nucleosomes throughout the cell cycle. What are the functional consequences of constitutive histone acetylation? Inactivation of the Sin3p/Rpd3p complex allows *HO* expression in the absence of *SWI5*, *SWI*/*SNF*, or *GCN5*; however, *sin3* or *rpd3* mutants still require *SWI4* and *SWI6* (Nasmyth et al. 1987; Sternberg et al. 1987; Stillman 1994; Wang et al. 1994; Perez-Martin and Johnson 1997). Furthermore, if a daughter cell inherits a highly acetylated *HO* locus, the Ash1p protein is unable to repress transcription in G_1. Consequently, *HO* is expressed in both mother and daughter cells in *sin3* mutants. (Nasmyth et al. 1987; Sternberg et al. 1987). This suggests that deacetylation of the *HO* locus is normally required for repression of *HO* by Ash1p in daughter cells.

Regulation of histone acetylation also appears to be required for control of *HO* expression during the mating process. If MAT**a** cells are exposed to the mating pheromone, α factor, the Cdc28p/Clnp kinase is inactivated, cells arrest in the G_1/S phase of the cell cycle, and *HO* is not expressed. When wild-type cells are released from the α factor block, *HO* is not expressed until the next cell cycle. The repression of *HO* in the first cell cycle following release probably ensures that the MAT**a** cell has one additional opportunity to mate with a nearby MATα partner before the mating type is switched by the *HO* endonuclease in the next cell cycle. However, in the absence of a functional Sin3p/Rpd3p deacetylase complex, *HO* is expressed immediately after release from the α factor block (Nasmyth et al. 1987). Thus, the functional interplay between histone acetylases (e.g., Gcn5p) and deacetylases (e.g., Rpd3) is crucial for several developmental controls of *HO* expression.

Another puzzling question concerns how the size of the acetylated histone domain is controlled. Previous studies have suggested that acetylation (Kuo et al. 1998) or deacetylation (Kadosh and Struhl 1998) might be limited to a region of only one or two nucleosomes. In these

cases, a HAT or HDAC might be targeted to a single site, in which it can only act on nucleosomes within reach. However, at *HO*, a *GCN5*-dependent complex acetylates perhaps six or seven nucleosomes, and the cell cycle timing of acetylation at different positions in the promoter suggests that Gcn5p might be able to move from its targeting site. Furthermore, we find that acetylation does not extend upstream of the SCB elements or downstream of the TATA region. In the latter case, the 5′ ORF probe does not detect a G_1 peak of acetylation even though it is only 200 bp from the TATA region in which the strong G_1 peak is detected. In fact, H3 acetylation at the *HO* coding region is much less than the level observed throughout the genome (cf. Figs. 1C and 2B). We believe it is unlikely that Swi5p or Swi4/Swi6p activators directly contribute to establishing the size of the acetylated domain, because the ~1-kb domain is established in a *swi4* deletion mutant, and Swi5p does not appear to remain stably associated with the *HO* locus during G_1 (Cosma et al. 1999). We favor a model in which boundary elements might exist in the *HO* promoter that either sequester a deacetylase or prevent the escape of a Gcn5p-containing HAT from the desired target region. One type of boundary might consist of TFIID, bound to the TATA element, which might restrain the Gcn5p-containing SAGA complex via physical interactions with one or more SPT subunits. Alternatively, a recent study (Pikaart et al. 1998) proposed that one possible function of insulator elements is to sequester acetylases or to exclude deacetylases. Consistent with this model, we have shown here that inactivation of the Sin3p/Rpd3p complex leds to an expanded domain of H3 acetylation that encompasses the entire *HO* locus.

Dependent chromatin remodeling events

Many of the genes whose expression requires SWI/SNF also rely on the activity of the Gcn5p HAT (Pollard and Peterson 1997). Previously, we proposed that Gcn5p acetyltransferase activity might control the ability of SWI/SNF to function on compacted nucleosomal arrays (Pollard and Peterson 1998). However, we have shown here that at the *HO* locus, SWI/SNF appears to be required at an early step that facilitates the recruitment or activity of the Gcn5p-containing HAT complex. It also possible that SWI/SNF functions at more than one step during the activation of *HO*. For instance, SWI/SNF may facilitate the binding of Swi4p/Swi6p to acetylated, nucleosomal sites. In this case, the prior acetylation of nucleosomes that encompass the Swi4p/Swi6p-binding sites may control the activity of SWI/SNF. In support of this view, the histone amino-terminal domains and the state of histone acetylation does alter the ability of SWI/SNF to function catalytically in vitro (Logie et al. 1999). Thus, although our analyses of the transitions in *HO* chromatin structure have uncovered one functional coupling between two distinct remodeling enzymes, it seems likely that the cell will have evolved multiple scenarios to ensure precise transcriptional regulation.

Materials and methods

Strains and medium

Strains were grown in YEP medium (2% yeast extract, 1% bactopeptone) containing either 2% glucose or 2% galactose. Most yeast strains used in this study are isogenic derivatives of the YPH strain set described by Sikorski and Hieter (1989). Deletion of *ASH1* was accomplished by a one-step gene disruption and was verified by Southern blot. Our isogenic set has the following relevant genotypes: *SWI*$^+$ *ash1Δ* (CY727); *gcn5Δ ash1Δ* (CY729); *swi2Δ ash1Δ* (CY725); *swi5Δ ash1Δ* (CY728); *swi4Δ ash1Δ* (CY726); *sin3Δ ash1Δ* (CY755).

Cell cycle synchronization and ChIPs

To arrest cells in G_2/M, nocodazole (Sigma, 15 µg/ml in DMSO) was added directly to the medium to a final concentration of 15 µg/ml. Cells were arrested for ~2 hr until arrest was at least 90% complete as determined by examination under the microscope. Cells were pelleted and washed in water, then resuspended in nocodazole-free medium. Samples were taken every 15 min and cross-linked with 1% formaldehyde for 15 min at room temperature. Cross-linking was quenched with 125 mM glycine and whole cell extracts were prepared for use in ChIPs. ChIPs were performed as described previously (Strahl-Bolsinger et al. 1997; Kuo et al. 1998). The average size of fragmented chromatin was ~500 bp. All ChIP experiments presented in this manuscript were performed at least four times—immunoprecipitations were performed two times for each of two independent cell synchronizations.

Probes and RNA analysis

All DNA hybridization probes used in these studies were generated by PCR and labeled by random priming. The probes (with their endpoints shown in parentheses relative to the start site of *HO* transcription) were amplified with the following primers: HO TATA (–227:–25), 5′-gcgtcacgaaaaaagaaatcaatcctacacagg-3′ and 3′-cgagatttaggtataggagtattcgtcg-5′; SCB1 (–950:–757), 5′-ccaaacttattacttttcctatttgaggttgg-3′ and 3′-gcttttcactagagctatgttaatctcctagg5′, SCB2 (–659:–461), 5′-gcgtcacgaaaaaagaaatcaatcctacacagg-3′ and 3′-ggacaaagatacggaaagagaatcattaagtgc-5′, 5A (–1386:–1144), 5′-ccttttttcatttttccctacgctcagggc-3′ and 3′-cccggcttagcgcatttttcaaactaagcacc-5′; 5B (–1847:–1637), 5′-cgtaaaattgtgcctttggacttaaaatggcg-3′ and 3′-ccattagaaacaatccacgctaaaataggc-5′; 3′ ORF (+720:+947), 5′-ggagagtttaagagaaaatgcgaaaatctgg-3′ and 3′-cctctcttcgtttagggacttaaatacagcc-5′; 5′ORF (+3:+233), 5′-gctttctgaaaacacgactattctgatggc-3′ and 3′-gggtccgcatcttgtcaaatagtcgcagaacg-5′. The GCS1 probe was amplified with 5′-ggaaagttcaattcacattgtagctataatgg-3′ and 3′-gggtagccgcaacaacatgacaacgttccg-5′; and the SSB1 probe was amplified with 5′-ccatttttagacttttttcttaactagaatgc-3′ and 3′-gcgctttctttttgtttatataaattaacac-5′.

HO transcription was detected by primer extension. Total RNA was prepared from cells by a glass bead lysis and phenol extraction method (Peterson and Herskowitz 1992). A total of 10 µg of RNA was hybridized with 1 ng of kinase-labeled oligonucleotide in 250 mM KCl at 50°C for 1 hr. Primers were extended with AMV reverse transcriptase (Promega) in 50 mM Tris (pH 8.0), 5 mM MgCl, 100 µg/ml actinomycin D, 10 mM DTT, 25 mM EDTA, and 0.25 mM dNTPs for 30 min at 42°C. Extension products were separated on an 8% acrylamide/8 M urea denaturing gel, and gels were fixed with 5% TCA, dried, and exposed to X-ray film.

Acknowledgments

We thank Kim Nasmyth for providing strains and for sharing unpublished results, Anita Sil and Ira Herskowitz for providing the ASH1 knockout plasmids, and Colin Logie, Mike Samuels, and other members of the Peterson laboratory for critical reading of the manuscript. This research was supported by grants from the National Institutes of Health (GM49650) to C.L.P. and the American Cancer Society to J.E.K. C.L.P. is a Scholar of the Leukemia Society of America.

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