Acetylation of H2AZ Lys 14 is associated with genome-wide gene activity in yeast

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Histone variants and their post-translational modifications help regulate chromosomal functions. Htz1 is an evolutionarily conserved H2A variant found at several promoters in the yeast *Saccharomyces cerevisiae*. In this study, we undertook a genome-wide analysis of Htz1 and its modifications in yeast. Using mass spectrometric analysis, we determined that Htz1 is acetylated at Lys 3, Lys 8, Lys 10, and Lys 14 within its N-terminal tail, with K14 being the most abundant acetylated site. ChIP and microarray analysis were then used to compare the location of Htz1-K14 acetylation to that of Htz1 genome-wide. The data presented here demonstrate that while Htz1 is associated preferentially with the promoters of repressed genes, K14 acetylation is enriched at the promoters of active genes, and requires two known histone acetyltransferases, Gcn5 and Esa1. In support of our genome-wide analysis, we found that the acetylatable lysines of Htz1 are required for its full deposition during nucleosome reassembly upon repression of *PHO5*. Since the majority of Htz1 acetylation is seen at active promoters, where nucleosomes are known to be disassembled, our data argue for a dynamic process in which reassembly of Htz1 is regulated by its acetylation at promoters during transcription.

[*Keywords*: Histone variants; H2AZ; Htz1; histone acetylation]

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Histone modifications have been implicated in regulating processes as diverse as gene expression (Kurdistani et al. 2004), apoptosis (Ahn et al. 2005), replication timing (Vogelauer et al. 2002), and responses to DNA damage (Vidanes et al. 2005), and act by modifying the properties of the nucleosomes to which they are attached. This can involve altering histone interactions with DNA and adjacent nucleosomes, ultimately affecting the higher-order folding of chromatin (Tse et al. 1998; Anderson et al. 2001), or the creation of binding sites for nonhistone chromosomal proteins (Dhalluin et al. 1999; Jacobson et al. 2000; Lachner et al. 2001).

A mechanistically distinct way to alter the chemical components, and therefore the properties, of a nucleosome is through the incorporation of variant histone proteins. Apart from the major H3 proteins, only two H3 variants are present in most lineages—a centromere-specific protein, cenH3, and a replication-independent replacement histone, H3.3 (Malik and Henikoff 2003). In contrast, there are many different H2A variants, including H2AZ, H2AX (West and Bonner 1980), H2AvD (a variant sharing H2AZ and H2AX properties, unique to *Drosophila*) (van Daal et al. 1988), macroH2A (Pehrson and Fried 1992), and H2A-Bbd (Chadwick and Willard 2001).

The study of histones and histone modifications at the whole genome level has been pioneered in the yeast *Saccharomyces cerevisiae*, whose small genome has facilitated genome-wide analyses (Bernstein et al. 2002; Robyr et al. 2002). The *S. cerevisiae* genome encodes one H2A variant, Htz1, which is a member of the highly conserved family of H2AZ variants (Jackson et al. 1996). Although H2AZ can take the place of H2A in *Xenopus* nucleosomes in vitro (Suto et al. 2000), Htz1 and H2A proteins cannot substitute for each other in yeast cells (Kolodrubetz et al. 1982; Jackson and Gorovsky 2000), and a dedicated histone replacement complex, SWR-C, has evolved to deposit Htz1 into chromatin (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004). The precise role of Htz1 in transcriptional regulation has remained elusive, although ~5% of yeast genes are misregulated in *htz1*Δ strains (Meneghini et al. 2003). Studies of individual genes have shown that Htz1 binds to the repressed promoters of several inducible genes, including *GAL1* and *PHO5*, but is lost from these promoters during gene activity (Santisteban et al. 2000; Adam et al. 2001), most likely in concert with the partial loss of nucleosomes from promoters that occurs upon activa-

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tion (Boeger et al. 2003; Reinke and Horz 2003; Schwabish and Struhl 2004). In addition, Htz1 in euchromatin is required to prevent the spread of yeast heterochromatin (Meneghini et al. 2003).

Interestingly, the SWR complex that deposits Htz1 into chromatin shares four of its subunits with the NuA4 histone acetyltransferase (HAT) complex (Kobor et al. 2004; Krogan et al. 2004). However, it is not known whether the NuA4 complex is involved in acetylation of Htz1 or whether Htz1 is post-translationally modified at all. Therefore, we have set out to determine by mass spectrometry the extent to which Htz1 is modified post-translationally in *S. cerevisiae* and to study the in vivo roles of any modifications. We find that Htz1 is acetylated in vivo at four N-terminal lysines (K3, K8, K10, K14) and have generated highly specific antibodies to the major acetylation site K14 and to the unmodified C terminus of Htz1. Using these reagents we compared the distributions of bulk Htz1 and acetylated Htz1 across the yeast genome. We find that Htz1 is present predominantly at inactive genes, where it is significantly enriched over promoters, while acetylated Htz1 is found at transcriptionally active genes that are undergoing extensive nucleosome loss. Htz1-K14 acetylation is dependent on the function of two HATs, Gcn5 and the NuA4 catalytic subunit Esa1. This argues that the SWR-C assembly complex and NuA4, in addition to sharing components, are linked through their actions on Htz1. We found that the N-terminal lysines of Htz1 are required for full deposition of this variant during nucleosome reassembly upon repression of *PHO5*. Since the majority of acetylation is seen at active promoters, where nucleosomes are being actively displaced and reassembled, our data argue for a dynamic process in which reassembly of Htz1 is regulated by its acetylation at promoters during transcription.

Results

The N-terminal tail of Htz1 is acetylated at four lysine residues

We first wanted to know whether H2AZ is modified post-translationally in yeast. Mass spectrometric analysis of Htz1 revealed that lysines in the N-terminal tail, at positions 3, 8, 10, and 14, are acetylated (Fig. 1). Htz1 was separated from the major core histones by HPLC (Fig. 1A) and treated with a deuterated acetylating agent. Unmodified lysines in the protein are acetylated by this treatment and can be distinguished from acetyl-lysines formed in vivo because of the mass difference between protiated and deuterated acetyl groups (Smith et al. 2003). After digestion with trypsin, electrospray ionization mass spectrometry (ESI-MS) analysis showed five ions, containing zero to four deuterated acetyl groups, within a peptide corresponding to the first 20 residues of Htz1 (Fig. 1B). Since there are four lysines within this sequence, this indicates that Htz1 can be acetylated at all of the lysines in the N-terminal tail.

To verify the identity of the acetylated sites, purified Htz1 was treated with trypsin, and the resulting peptides were isolated and subjected to tandem mass spectrometry (MS/MS). Peptides whose spectra include the immonium ion at mass-to-charge ratio (*m/z*) 126.1 U that is a signature of lysine acetylation are shown in Figure 1, C and D. We found a peptide acetylated at Lys 14 (Fig. 1C), and a peptide carrying acetylation at both Lys 10 and Lys

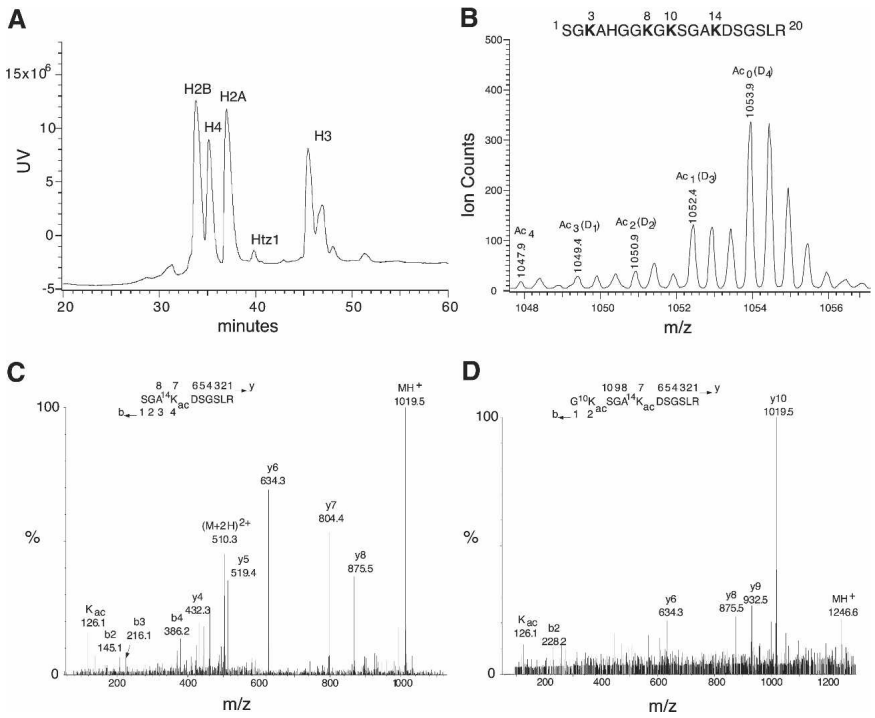

Figure 1. Mass spectrometric analysis of Htz1 identifies acetylation on four lysines in the N-terminal tail. (*A*) Histones isolated from asynchronously growing yeast cells were separated by RP-HPLC (C4 column). Htz1 is eluted after H2B, H4, and H2A but before H3. (*B*) The ESI-MS of peptides covering the sequence 1–20 from Htz1 treated with D_6-acetyl anhydride and digested with trypsin. Doubly charged ions at *m/z* 1047.9, 1049.4, 1050.9, 1052.4, and 1053.9 correspond to the peptide with sequence Ac[1]SGKAHGGKGKSGAKDSGSLR that is un-, mono-, di-, tri-, and tetra-acetylated at K3, K8, K10, and K14, respectively. (*C*) ESI/MS/MS was performed on the ion at *m/z* 1019.6 (observed doubly charged ion at *m/z* 510.3) to verify its identity. The spectrum shows singly charged b-type and y-type ions corresponding to the sequence shown, in which Lys 14 is acetylated. (*D*) ESI/MS/MS was performed on the ion at *m/z* 1246.6 (observed doubly charged ion at *m/z* 623.8) to verify its identity. The spectrum shows singly charged b-type and y-type ions matching the sequence shown, in which both Lys 10 and Lys 14 are acetylated.

14 (Fig. 1D). We were unable to verify the acetylation at Lys 3 and Lsy 8 by this analysis because we did not recover peptides including these sites. A calculation based on the ion intensity distribution of the five ions and the fragmentation ions from the MS/MS spectrum indicates that acetylation at K3, K8, K10, and K14 is present on roughly 3%, 7%, 14%, and 38% of the Htz1 molecules, respectively. Our findings indicate that the N terminus of Htz1 is acetylated predominantly at Lys 14, and to a lesser extent at Lys 3, Lys 8, and Lys 10.

Comparison of bulk Htz1 binding and K14 acetylation across the yeast genome

To compare the distributions of bulk and acetylated Htz1 on a genome-wide scale, we used a chromatin immunoprecipitation (ChIP) and microarray approach. First, we raised antibodies to a synthetic Htz1 N-terminal peptide acetylated at K14 and to a C-terminal unmodified peptide (Supplementary Fig. S1A). The C terminus of Htz1 was chosen as an antigen to generate antibodies to bulk Htz1 since it is the region most dissimilar to H2A and because we did not identify any modifications in this region in our mass spectrometric analysis. We chose to examine K14 over the other acetylation sites because this is the most abundant site of acetylation (Fig. 1C,D). In addition, we could exclude the other acetylatable lysines in our peptide antigen to avoid raising antibodies dependent on acetylation status at more than one site.

For each antigen, crude antisera from eight rabbits were screened by ELISA to identify those that specifically bound to their target antigen in the presence of competitor peptides. The competitor peptides were a C-terminal H2A peptide in the case of the Htz1 C-terminal antisera, and an unacetylated N-terminal peptide in the case of the Htz1-K14Ac antisera (Supplementary Fig. S1B,D). The sera that showed the greatest specificity for each of the two antigens by ELISA were selected and affinity-purified. In vivo specificity in ChIP was then checked using lysates from yeast strains genetically modified to lack the desired epitope (amino acid substitutions at Htz1-K14 that prevent acetylation, or deletion of Htz1). In each case, the affinity-purified antibodies could precipitate Htz1 and cross-linked DNA only from cells containing the wild-type antigen (Supplementary Fig. S1C,E). We had therefore obtained antibodies highly specific for Htz1 acetylated at K14 (antibody α741) and for bulk Htz1 (antibody α660).

Using these new tools, we asked where Htz1 and K14-acetylated Htz1 are distributed across the 16 yeast chromosomes. Antibodies α660 and α741 were used in ChIP to isolate Htz1- or Htz1-K14Ac-associated DNA fragments. The purified DNA fragments were then compared to input DNA by competitive hybridization to whole-genome microarrays. These microarrays are spotted with probes corresponding to RNA polymerase II-transcribed ORFs and to intergenic regions (IGRs). Although the genome-wide binding profile of Htz1 has recently been reported (Guillemette et al. 2005; Raisner et al. 2005; Zhang et al. 2005) (while this manuscript was in preparation), this experiment is a critical control for our study of acetylated Htz1 and we include our data on Htz1 binding here.

Htz1 is enriched on inactive genes, but K14 is hyperacetylated on active genes

To ascertain whether the set of genes that are bound preferentially by Htz1 fall into a biologically cohesive class, we queried the Functional Specification database (Robinson et al. 2002). We found genes encoding mitochondrial ribosome subunits ($p = 7.94 \times 10^{-6}$), genes coding for vitamin metabolism enzymes ($p = 6.78 \times 10^{-6}$), and a large number of genes whose function is unknown ($p = 1.53 \times 10^{-7}$) among the genes most enriched for Htz1. These gene classes have a feature in common—they are repressed under the growth conditions (rich medium; rapidly dividing cells) that we have used for our analysis. To ask whether Htz1 is generally enriched on less active genes, we directly compared the binding of Htz1 to ORFs and promoters to the transcriptional frequencies of the corresponding genes (Holstege et al. 1998). As Figure 2 shows, Htz1 binding to both ORFs (correlation value [r] = –0.23) (Fig. 2A) and promoter IGRs (r = –0.26) (Fig. 2B) is markedly anticorrelated with transcription, indicating that Htz1 is more abundant on less active genes.

To ask how Htz1-K14 acetylation levels relate to gene activity, we compared genome-wide K14 acetylation to transcriptional frequencies. Visual inspection of this comparison reveals a positive trend for Ht1z-K14 acetylation on ORFs (Fig. 2C), but the correlation is low (r = 0.03), and there is similarly little or no correlation between IGR acetylation and transcriptional frequency (r = –0.09) (Fig. 2D). However, our analysis of Htz1 binding showed that Htz1 tends to be lost from very active genes, which means that by looking at the raw data we are underestimating the correlation between gene activity and Htz1 acetylation. Normalization of the Htz1-K14Ac data to the abundance of bulk Htz1 results in a very strong correlation with active genes (r = 0.51) (Fig. 2E) and promoters (r = 0.37) (Fig. 2F). Another demonstration that Htz1 is hyperacetylated on active coding regions comes from querying the Functional Specification database, which shows that genes hyperacetylated at Htz1-K14 include those involved in ribosome biogenesis ($p < 10^{-14}$) and protein synthesis ($p < 10^{-14}$). Such genes are among the most highly expressed in *S. cerevisiae* (Warner 1999). Indeed, we see genes from these categories among the most acetylated ORFs (top 200) even before we normalize to bulk Htz1 occupancy (p = 0.001). Therefore, active genes are clearly enriched for Htz1-K14 acetylation.

Htz1-K14 acetylation is correlated with other "active" acetylation sites

In yeast, active loci carry core histones with a characteristic pattern of acetylation, with hyperacetylation of H3-

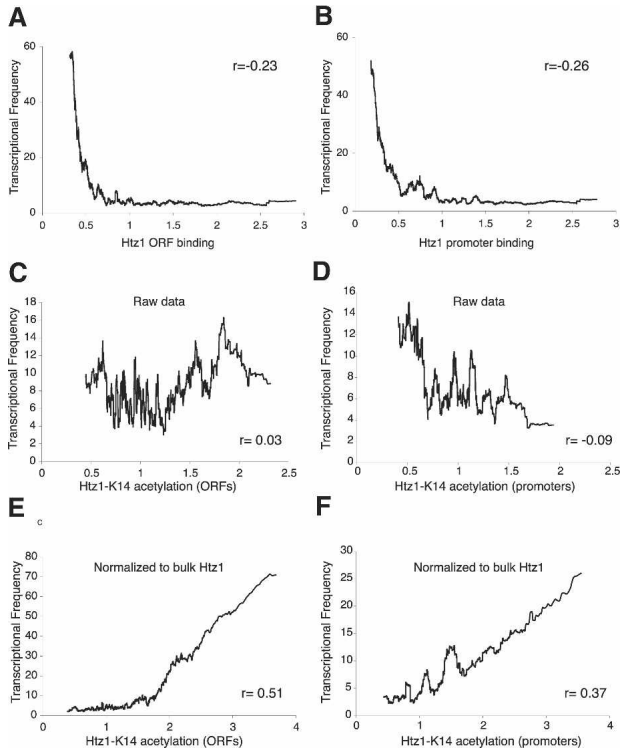

Figure 2. Htz1 is depleted but hyperacetylated at active genes. (*A*,*B*) Moving averages (window size 100) of Htz1 binding and transcriptional frequencies at ORFs (*A*) and IGRs (*B*) are plotted relative to each other. The transcriptional frequencies for IGRs are the values for the cognate ORF. The Pearson correlation values (*r*) for the data sets are indicated. (*C*–*F*) Moving averages (window size 100) of Htz1-K14 acetylation at ORFs (*C*,*E*) or IGRs (*D*,*F*) are plotted relative to transcriptional frequency. The raw data are plotted in *C* and *D*; in *E* and *F*, the acetylation data have been normalized to bulk Htz1 occupancy. The Pearson correlations (*r*) are shown in each panel.

K18 and hypoacetylation at H4-K16 being two hallmarks of gene activity (Kurdistani et al. 2004). In pairwise comparisons between our Htz1-K14 acetylation data and our previous data on acetylation at other core histone lysines (Kurdistani et al. 2004; Xu et al. 2005), we found positive correlations with acetylation on the other histones (Fig. 3). Htz1-K14 acetylation correlates poorly with acetylation at H3-K56, a cell cycle-specific modification that is involved in histone gene regulation and the DNA damage response (Masumoto et al. 2005; Xu et al. 2005), indicating that the function of Htz1-K14 acetylation is likely to be distinct from that of H3-K56. The other notably low correlation is to H4-K16Ac. Acetylation at this site is anticorrelated with transcription (Kurdistani et al. 2004; Liu et al. 2005); therefore, the low correlation of Htz1-K14Ac with H4-K16Ac, coupled with high correlation to H3 sites, is consistent with Htz1-K14 acetylation at active genes.

Htz1 and Htz1-K14 acetylation are enriched on promoters

Although Htz1 appears similar to other core histones with respect to its general depletion from active genes, the profile of Htz1 binding shows a surprising feature. While others have observed a higher occupancy of major core histones on ORFs than on IGRs (Lee et al. 2004; Sekinger et al. 2005), we find that the opposite is true for Htz1 (Fig. 4A). Ten percent of genes have twofold or greater more Htz1 bound to the promoter than the ORF, while the core histone H4 shows comparable promoter enrichment at only 0.5% of genes (Sekinger et al. 2005). Htz1 is therefore generally enriched on IGRs rather than on their corresponding ORFs. To test whether Htz1 is specifically associated with gene promoters or if it merely has a general preference for noncoding regions, we analyzed the binding of Htz1 with respect to different IGR classes. The IGR fragments present on our microarrays include promoters of single genes, promoters of divergently transcribed genes (double promoters), as well as different types of non-promoter-containing fragments (telomeric or centromeric DNA; tRNA, snoRNA and snRNA genes, transposons, etc.) (see Table 1). The distribution of different IGR types in the Htz1 binding data showed that 96% of the IGRs that are most enriched for Htz1 (top 200) are promoters, whereas in the whole data set only 71.6% of the IGRs are promoters (Fig. 4B; Table 1). Conversely, the fragments that are depleted for Htz1 (bottom 200) have a dramatically reduced percentage of promoters (33.5%) and an increased percentage of non-promoter fragments from the whole-genome average of 19.4% to 52.5%. Therefore, not only is Htz1 preferentially bound to gene promoters ($p = 2.59 \times 10^{-20}$), but also fragments that are depleted for Htz1 are predominantly nonpromoter sequences.

Analysis of the abundance of K14 acetylation on pro-

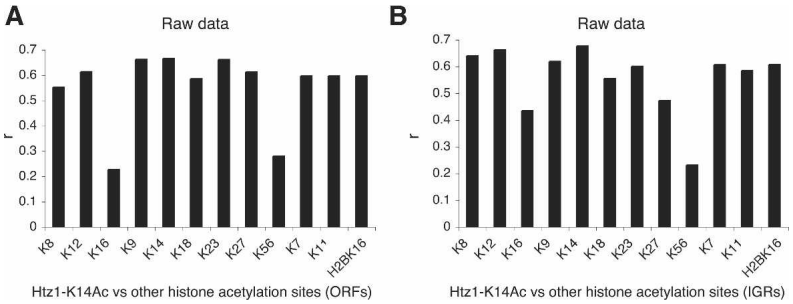

Figure 3. Htz1-K14 acetylation correlates positively with other histone acetylation sites. Correlation values (*r*) of Htz1-K14 acetylation with other core histone acetylation sites are displayed graphically. Data for ORFs are shown in *A* and for IGRs in *B*.

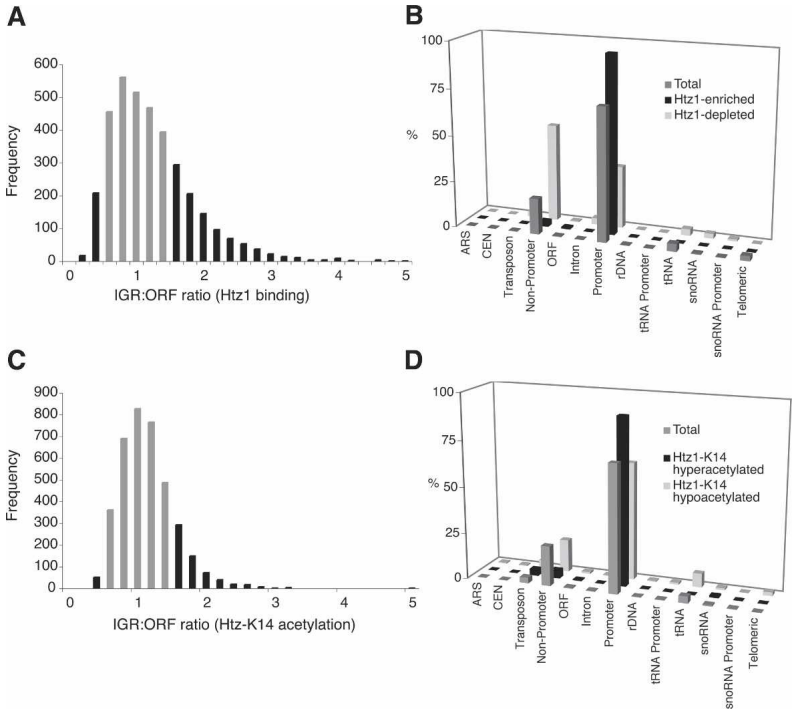

Figure 4. Htz1 and Htz1-K14 acetylation are enriched on promoters. (*A*) Htz1 is enriched on promoters relative to ORFs. Histogram plot of the ratios of Htz1 binding at promoter IGRs and their cognate ORFs (IGR:ORF ratios). Bars representing IGR:ORF ratios close to 1 are gray; bars representing ratios different from 1 are black. (*B*) Distribution of IGR classes within the total Htz1 data set (dark-gray bars) relative to the Htz1-enriched (black bars) and Htz1-depleted (light-gray bars) subsets. The abundance of each IGR subtype in each data set is expressed as a percentage of the total. (*C*) Histogram plot of the ratios of Htz1-K14 acetylation at promoter IGRs and their cognate ORFs (IGR:ORF ratios). Bars representing IGR:ORF ratios close to 1 are gray; bars representing ratios different from 1 are black. (*D*) Distribution of IGR classes within the total Htz1-K14 acetylation data set (dark-gray bars) relative to the Htz1-K14 hyperacetylated (black bars) and Htz1-K14 hypoacetylated (light-gray bars) subsets. The abundance of each IGR subtype in each data set is expressed as a percentage of the total.

moters relative to gene coding sequences shows that the distribution of K14-Ac at IGRs relative to ORFs is also skewed toward ratios >1 (Fig. 4C). The degree of enrichment of K14 acetylation on promoters relative to coding regions is not as profound as for Htz1, with 2.3% of genes having IGR:ORF ratios ≥2. This difference is most likely due to the preferential loss of promoter nucleosomes from active genes (Lee et al. 2004), where K14 is acetylated. Nevertheless, when we examine the distribution of IGR subtypes in the K14-hyperacetylated data (Fig. 4D; Table 2), we can see that 90.5% of the hyperacetylated IGR fragments are promoters compared with 69% in the whole data set, which is highly significant ($p = 3.4 \times 10^{-13}$). We conclude that Htz1 and Htz1-K14 acetylation are both enriched at promoters, although acetylation is enriched at the promoters of active genes.

Htz1 acetylation is preferentially excluded from the ends of yeast chromosomes

Since Htz1 has a role in preventing the spread of SIR proteins from subtelomeric heterochromatin into adjacent subtelomeric euchromatin (Meneghini et al. 2003), we were interested to examine how Htz1 and Htz1-K14 acetylation related to distance from telomeres. We mapped the chromosomal positions of Htz1-enriched and hyperacetylated fragments relative to the ends of chromosomes and found that while Htz1 is present at average levels near the end of chromosomes adjacent to subtelomeric heterochromatin (Fig. 5A), it remains hypoacetylated for up to 50 kb from the ends of chromosomes (Fig. 5B). The hypoacetylation of Htz1-K14 is also apparent from the distribution of IGR subtypes in the Htz1-K14Ac binding data (Fig. 4D; Table 2), where the least acetylated IGR fragments show a 20-fold enrichment for telomeric sequences ($p = 4.1 \times 10^{-5}$). Therefore, while Htz1 is present adjacent to subtelomeric chromatin, there is a broad domain of Htz1-K14 hypoacetylation up to 50 kb from the telomeres.

Table 1. *Distribution of IGR subtypes in the αHtz1 microarray data set*

IGR type	Total data set (%)	Htz1 enriched (%)	Htz1 depleted (%)
ARS	0.07	0	0
CEN	0.1	0	0
Transposon	0.2	0	3
Telomeric	2.6	0	0
ORF	0.4	1	0.5
Promoter	71.6	96	33.5
Intron	0.17	0	3.5
Nonpromoter	19.4	3	52.5
rDNA	0.3	0	0
tRNA	4.2	0	3.5
tRNA promoter	0.2	0	0
snoRNA	0.6	0	2.5
snoRNA promoter	0.2	0	1

Percentage representation of each IGR type is shown for the total data set, for the top 200 (Htz1-enriched) IGRs, and for the bottom 200 (Htz1-depleted) IGRs. Htz1 is enriched at promoters, and conversely, fragments depleted for Htz1 are predominantly nonpromoters. Htz1-enriched fragments are almost exclusively promoters, with telomeric and nonpromoter sequences representing 4% of the Htz1-enriched fragments. The ORFs that are part of the IGR data represent fragments of control genes; for example, *GAL1, INO1*.

Table 2. *Distribution of IGR subtypes in the αHtz1K14Ac microarray data set*

IGR type	Total data set (%)	Htz1-K14 hyperacetylated (%)	Htz1-K14 hypoacetylated (%)
ARS	0.08	0	0.5
CEN	0.1	0	0.5
Transposon	3	3.5	4.5
Telomeric	0.1	0	2
ORF	0.5	0.5	1
Promoter	69	90.5	63.5
Intron	0.3	0	0.5
Nonpromoter	21.6	4.5	17.5
rDNA	0.2	0	0.5
tRNA	3.8	0	7.5
tRNA promoter	0.3	0	1
snoRNA	0.7	1	1
snoRNA promoter	0.3	0	0

Percentage representation of each IGR subtype is shown for the total data set, for the top 200 hyperacetylated IGRs, and for the bottom 200 hypoacetylated IGRs. Htz1 is hyperacetylated at promoters, while telomeric sequences and tRNA genes are significantly hypoacetylated at K14.

Gcn5 and Esa1 are required for Htz1 acetylation

To ask whether any of the known yeast HATs are required for Htz1 acetylation, we systematically tested strains carrying inactivating mutations in these HATs for reduced Htz1 acetylation levels. In most cases, we used strains deleted for a particular HAT, but for Esa1, a HAT that is required for viability in yeast, we used a temperature-sensitive mutation and tested for acetylation after growth at the nonpermissive temperature (Clarke et al. 1999). We chose several genomic loci with relatively high levels of Htz1-K14 acetylation from the ChIP-microarray data and designed primers to these IGRs and ORFs to use in semiquantitative PCR analysis. This assay allowed us to control for bulk Htz1 abundance and additionally to check levels of other modifications that are known targets of particular HATs. Deletion of HATs, including Sas2, Sas3, Elp3, Hpa2, Hpa3, Nut1, and Hat1, had no apparent effect on Htz1-K14 acetylation (data not shown). In contrast, we found that Htz1 acetylation at K14 is decreased strongly in two mutants, *esa1^{ts}* and *gcn5*Δ (Fig. 6). A strain defective for both Gcn5 and Esa1 activities had only background levels of acetylation of Htz1, indicating that these two enzymes are directly or indirectly responsible for most, if not all, acetylation of Htz1-K14 in vivo.

Htz1 acetylation is important for deposition of this variant histone

To determine whether acetylation of Htz1 is important for a biological function in yeast, we mutated the acetylatable lysines in the N-terminal tail of Htz1. Amino acid substitutions (to arginine or glutamine) at each of the four acetylatable lysines, singly and in combination, as well as a deletion of the N-terminal tail of Htz1, were generated and integrated at the endogenous *HTZ1* locus. The resulting strains were tested, in parallel with an *htz1*Δ strain, to see whether lack of acetylation is equivalent to lack of Htz1. Although Htz1 is not required for viability when yeast are growing on rich medium containing glucose, *htz1*Δ cells grow poorly when galactose is the carbon source and are sensitive to several DNA-damaging agents and to microtubule-depolymerizing drugs (Adam et al. 2001; Larochelle and Gaudreau 2003; Kobor et al. 2004; Krogan et al. 2004; Mizuguchi et al. 2004). Substitutions at the acetylatable lysines or complete deletion of the N-terminal tail did not recapitulate the *htz1*Δ phenotype on galactose, HU, benomyl, caffeine, or formamide. In fact, all of the mutant strains grew like wild-type cells on these media (data not shown). The function of Htz1 that is required for growth on these media is therefore not dependent on the acetylatable lysines.

In addition to growth defects on certain media, cells lacking Htz1 have altered patterns of gene expression and show increased spreading of SIR silencing proteins into euchromatin (Meneghini et al. 2003). We found no evidence of altered transcription in strains lacking Htz1 acetylation, nor did we see altered Sir3 abundance at *HMR* (data not shown). It should be noted, however, that even deletion of *HTZ1* produces very small changes in Sir3 binding in our strain background (YDS2). Others have noticed a similar phenomenon in the W303 background (Tackett et al. 2005). Since acetylation of K14 is low throughout the telomere-proximal 50 kb of yeast chromosomes, we believe that it is unlikely that acetylation of Htz1 plays a direct role in telomeric silencing.

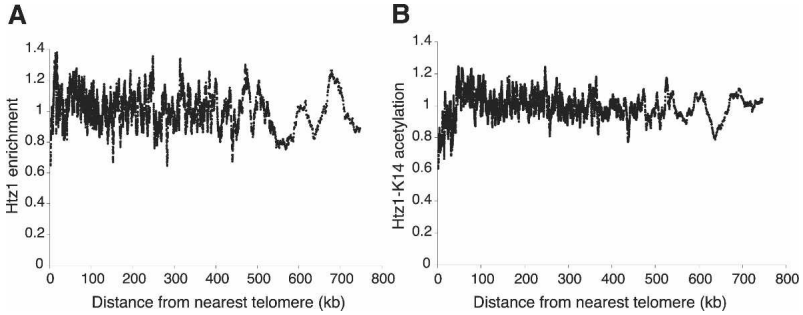

Figure 5. Htz1 distribution relative to chromosomal position. (*A*) Htz1 enrichment is plotted against distance to the nearest telomere (moving averages; window size 50). (*B*) Htz1-K14acetylation levels are plotted relative to distance from the nearest telomere (moving averages; window size 50).

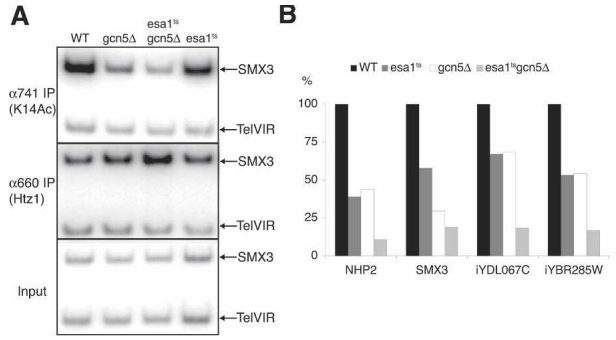

Figure 6. Gcn5 and Esa1 are redundantly required for Htz1-K14 acetylation. Levels of Htz1-K14 acetylation were determined by ChIP using α741 antibodies; α660 antibodies were used in ChIP to control for bulk Htz1 levels. Enrichments relative to a fragment 0.5 kb from the end of chromosome VIR (TelVIR) were calculated and normalized to input DNA. (*A*) Semiquantitative multiplex PCR analysis of Htz1-K14 acetylation (*top*), bulk Htz1 (*middle*), and input DNA (*bottom*) at *SMX3*. Inactivating mutations in either Esa1 or Gcn5 reduce the enrichment of Htz1-K14Ac at *SMX3*, but a double mutation reduces acetylation to background levels. Levels of Htz1 are shown for comparison. Increased levels of Htz1 in the HAT mutant strains correspond to reduced transcription (as measured by Pol II enrichment), but mutations in other HATs that caused reduced Pol II enrichment did not show loss of Htz1-K14 acetylation (data not shown). (*B*) Quantitation of ChIP data for four loci (two ORFs and two IGRs). K14 acetylation is displayed as a percentage of the wild-type level (WT) and has been normalized to total Htz1 occupancy. The mean values from three independent ChIPs with α741 (Htz1-K14Ac), divided by the mean values from ChIPs with α660 (bulk Htz1), are plotted. Error bars are not indicated because the data represent divided ratios, but variation between experiments was negligible.

To test whether acetylation is required for Htz1 deposition into chromatin, we initially looked for differences in Htz1 enrichment in chromatin by ChIP and microarray analysis. We directly compared Htz1 chromatin binding in a wild-type (WT) strain to binding in a strain in which the acetylated lysines in the Htz1 N terminus were mutated to arginine (4K-R) by cohybridizing DNA fragments isolated by αHA-Htz1 ChIP from each strain to the same microarray. Examination of the loci with the greatest deviation from the mean 4K-R:WT ratios showed a relationship to transcriptional activity. The average transcriptional frequency of the top 50 genes (i.e., where there is a high 4K-R:WT ratio) is 39 mRNAs per hour, whereas the average for the bottom 50 genes is five mRNAs per hour. This indicates that 4K-R mutant Htz1 is preferentially absent from repressed genes, where levels of Htz1 are normally high.

While the microarrays described above proved useful for identifying promoters with altered Htz1 ratios in the absence of acetylation, we wished to analyze Htz1 deposition in a time course of activation and repression of a specific gene. We chose *PHO5* for our analysis, since our array data indicated a reduced amount of Htz1 in the absence of acetylation at the promoter of *PHO5* and because this gene is well studied and its expression can be easily manipulated. Using ChIP followed by semiquantitative PCR with primers directed to the *PHO5* promoter (Fig. 7, top; Reinke and Horz 2003), we examined the relative levels of wild-type and 4K-R Htz1, and their kinetics of loss during gene activation and reassembly during repression. Very similar findings were made for nucleosomes-1 and -2 at the *PHO5* promoter, so we show only the data for nucleosome-2. We find a dramatic difference (approximately threefold) between levels of wild-type and 4K-R Htz1 at the *PHO5* promoter under repressed conditions (Fig. 7A, 0 min). As *PHO5* is activated in low phosphate medium, both wild-type and 4K-R Htz1 are lost from the promoter, with levels reaching the same baseline in both strains. In contrast, overall nucleosome loss, as measured by H3 levels, is the same in both strains (Fig. 7B). During the transition from activation to repression, wild-type Htz1 is assembled into chromatin to a higher level than the unacetylatable protein, while histone H3 abundance is relatively similar in both strains (Fig. 7C,D). Htz1 assembly occurs surprisingly rapidly (50% of the maximal incorporation of Htz1 after only 1 min) (Fig. 7E). Mutating the N-terminal lysines to glutamine instead of arginine resulted in a similarly reduced level of Htz1 incorporation at the *PHO5* promoter (data not shown), arguing for the importance of the acetylation sites of Htz1 in its assembly.

A possible alternative explanation for reduced Htz1 levels at a promoter is increased transcription from that promoter. However, RT–PCR analysis of *PHO5* expression during activation and repression revealed no obvious difference between the wild-type and 4K-R strains (Supplementary Fig. S2A,B), indicating that reduced Htz1 binding in the 4K-R strain is not a result of increased *PHO5* transcription. Another potential cause of the reduced Htz1 level in the 4K-R mutant is reduced *HTZ1* expression, but RT–PCR analysis of *HTZ1* expression revealed that the level of *HTZ1* mRNA is not lowered in the 4K-R mutant strain (Supplementary Fig. S2C,D). Furthermore, Western blot analysis of Htz1 in wild-type and 4K-R strains revealed indistinguishable protein levels (data not shown), so that the reduced deposition of Htz1 into chromatin is not due to a decrease in Htz1 abundance. Since Htz1-K14 is acetylated at the *PHO5* promoter during gene activity (Fig. 7F) and the substitution of the acetylation sites results in decreased levels of 4K-R Htz1 at this promoter, we conclude that acetylation of Htz1 is important for its deposition.

Discussion

A detailed map of core histone binding and histone post-translational modifications across the yeast genome is beginning to emerge from various laboratories (Kurdistani et al. 2004; Lee et al. 2004; Liu et al. 2005; Pokholok et al. 2005; Sekinger et al. 2005). This type of information is a valuable first step in understanding how portions of the genome are specialized for different functions. A complete picture of the nucleosomal landscape will not only include information on post-translational

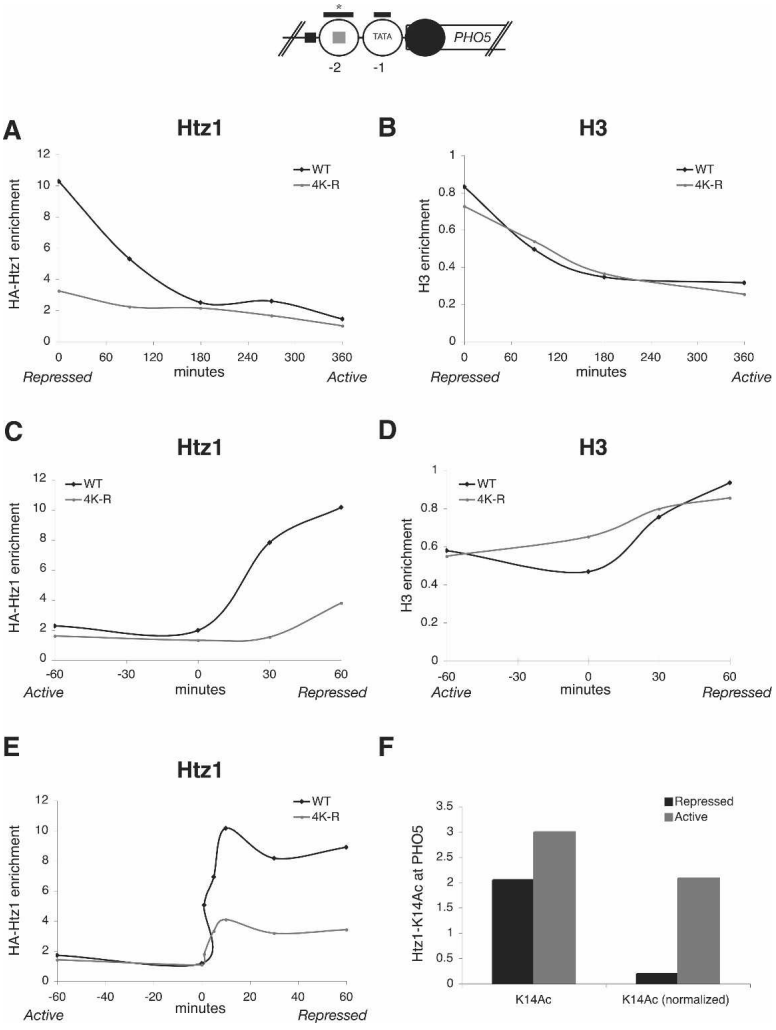

Figure 7. Acetylation regulates deposition of Htz1 at the *PHO5* promoter. Levels of HA-Htz1, H3, and Htz1-K14 acetylation at the *PHO5* promoter were determined by ChIP. Quantitations of enrichments at *PHO5* relative to TelVIR are plotted. The schematic diagram (*top*) of the *PHO5* promoter shows promoter nucleosomes as open circles and the 5′ ORF nucleosome as a filled circle, with UASp1 (black box), UASp2 (gray box), and the TATA box also shown. The positions of the primer sets used for PCR amplification are indicated by thick black lines *above* nucleosomes –1 and –2. An asterisk indicates the primer set for which the data are shown, although both primer sets gave identical results. (*A*) HA-Htz1 binding in cells that have wild-type (WT; black line) or mutated (4K-R; gray line) Htz1. At 0 min the cells were shifted from high- to low-phosphate media. (*B*) H3 binding in the Htz1-WT (black line) or Htz1-4K-R (gray line) strains. The cells were shifted from high- to low-phosphate media at 0 min. (*C*) HA-Htz1 binding in cells that have wild-type (WT; black line) or 4K-R (gray line) Htz1. At –60 min the cells were growing in low-phosphate medium; at 0 min phosphate was added. (*D*) H3 binding in wild-type (WT; black line) or 4K-R (gray line) strains. At –60 min the cells were growing in low-phosphate medium; at 0 min phosphate was added. (*E*) HA-Htz1 binding in cells that have wild-type (WT; black line) or 4K-R (gray line) Htz1. At –60 min the cells were growing in low-phosphate medium; at 0 min phosphate was added and samples were taken at 1, 5, 10, 30, and 60 min after phosphate addition. (F) Htz1-K14 acetylation at the PHO5 promoter under repressed (black bars) or active (gray bars) conditions. Raw data are plotted to the *left*; the values to the *right* have been normalized to bulk Htz1 levels to account for nucleosome loss during gene activity.

modifications but must also characterize the distribution of nonstandard histones. Three very recent studies have described in detail the distribution of Htz1 in yeast (Guillemette et al. 2005; Raisner et al. 2005; Zhang et al. 2005). These studies, and our data presented here, show that Htz1 is preferentially bound at repressed promoters genome-wide. We also describe the acetylation sites of Htz1, the genomic distribution of the major acetylation site at Lys 14, the enzymes required for acetylation of Htz1-K14, and a role for the acetylation sites in Htz1 deposition.

Htz1 is acetylated at four lysine residues in the N-terminal tail

The amino acid sequence of Htz1 contains many potential sites of post-translational modification (lysines, arginines, serines, etc.) throughout its length. After trypsin digestion, we recovered peptides covering 70% of the sequence of Htz1, including the central histone fold domain and the C-terminal tail. However, mass spectrometric analysis showed acetylation only on K3, K8, K10, and K14 within the N-terminal tail. This does not rule out the presence of modifications in other regions of Htz1, since there are some sequences for which we did not recover any data; however, it indicates that the N-terminal tail of Htz1, like those of the other core histones, is a major target for regulation through acetylation and deacetylation. H2AZ acetylation is likely to be conserved in other species, since Ren and Gorovsky (2001) showed that mutating the N-terminal lysines of *Tetrahymena* H2AZ results in altered mobility in TAU gels, which is consistent with the wild-type protein carrying charged chemical modifications such as acetylation. In addition, while our manuscript was in preparation, Bruce et al. (2005) reported that chicken H2AZ is acetylated at several active chicken genes.

Htz1 is enriched on repressed promoters, while Htz1-K14 acetylation is found at active promoters

The observed enrichment of Htz1 at promoters genome-wide extends the observations of Santisteban et al. (2000) at the *GAL1* and *PHO5* genes and Larochelle and Gaud-

reau (2003) at *PUR5*, and agrees with recently published genome-wide studies (Guillemette et al. 2005; Raisner et al. 2005; Zhang et al. 2005). Not surprisingly, since Htz1 acetylation by definition requires Htz1 presence, Htz1-K14 acetylation is also preferentially found on promoter sequences. It should be noted that it is a known technical peculiarity of ChIP experiments that intergenic DNA is recovered less efficiently than coding sequences (Pokholok et al. 2005). Therefore, it is likely that even with the enrichment that we see, we are underestimating the promoter:ORF ratios of Htz1. Yeast promoters are generally poor in nucleosomes (Sekinger et al. 2005; Yuan et al. 2005), so it seems probable that most of the sparse promoter nucleosomes contain Htz1. The role of Htz1 at these sites remains unclear. It has been proposed that Htz1 may mark nucleosomes for eventual displacement (Zhang et al. 2005); however, no genetic evidence has been presented to support this view. Alternatively, Guillemette et al. (2005) recently showed that Htz1 is required for normal nucleosome positioning at the *GAL1* promoter, indicating that Htz1-containing nucleosomes are important directly or indirectly for promoter chromatin organization in yeast.

Despite the absence of clear roles for Htz1, there is reason to think that the role of Htz1 acetylation is more tractable. We find that bulk Htz1 and K14-acetylated Htz1 differ in their genome-wide distributions in a manner that relates to transcriptional activity. While Htz1, like the other core histones (Boeger et al. 2003; Reinke and Horz 2003; Bernstein et al. 2004; Lee et al. 2004), is depleted from active genes, several lines of evidence indicate that Htz1-K14 acetylation is enriched at active genes. First, genes that are highly transcribed are in the top percentiles for Htz1-K14 acetylation. Second, Htz1-K14 acetylation correlates well with H3-K18, known to be hyperacetylated on active genes, but poorly with H4-K16, which is underacetylated on active genes. Finally, Htz1-K14 acetylation shows a strong correlation with transcriptional frequency when the data are normalized to bulk Htz1 occupancy. Zhang et al. (2005) have shown that Htz1 is more easily dissociated from chromatin than is H2A by in vitro washing, and suggest that Htz1 facilitates nucleosome loss during gene activity. Since Htz1 is relatively depleted from active genes, where it is highly acetylated at K14, one possible role for K14 acetylation is in promoting the loss of Htz1 and entire nucleosomes. However, we found that mutating the acetylatable lysines in the Htz1 N-terminal tail did not alter the kinetics of Htz1 or H3 loss during activation of *PHO5*, indicating that the acetylation does not destabilize Htz1. It seems, therefore, that the acetylation of K14 does not cause Htz1 or nucleosome loss. Instead, as discussed below, Htz1 acetylation is likely to promote Htz1 deposition.

Htz1 acetylation requires known HATs and regulates chromatin deposition

Htz1 acetylation at K14 in vivo is drastically reduced in the absence of Esa1 and Gcn5. These HATs are the catalytic components of the NuA4 and SAGA complexes, subunits of which are known to interact genetically with Htz1 (Santisteban et al. 2000; Krogan et al. 2003; Kobor et al. 2004). NuA4 shares components with the Htz1 deposition complex SWR-C (Kobor et al. 2004), and in higher organisms the orthologs of Esa1 and Swr1 are part of a single complex (Doyon and Cote 2004; Doyon et al. 2004). Our finding that unacetylatable mutants of Htz1 are poorly deposited into chromatin supports a functional as well as physical connection between NuA4 and SWR-C and suggests that part of the assembly process of Htz1 involves its acetylation. Nevertheless, we cannot exclude the formal possibility that the Htz1 N-terminal lysines are important for deposition independently of their acetylation, for example, as part of a protein-binding site. An argument against this, however, is provided by the data of Zhang et al. (2005), who have shown that Htz1 deposition genome-wide is reduced in a *gcn5*Δ mutant. Their interpretation of this finding is that an altered H3 acetylation pattern fails to recruit the SWR-C; however, their data are consistent with our finding that Htz1 acetylation levels are reduced in *gcn5*Δ strains, which would lead to reduced deposition of Htz1. Therefore, we propose that the acetylation of Htz1 regulates the amount of Htz1 in chromatin.

Although the level of Htz1 is dramatically reduced at the *PHO5* promoter when the acetylation sites are mutated, there are clearly residual amounts of Htz1 present. Similarly, in the *esa1*^{ts}*gcn5*Δ mutant, where K14 acetylation is essentially abolished, we can detect Htz1 binding at the active genes we have studied. One explanation for this is that there is more than one pathway for the deposition of Htz1. Indeed, while this manuscript was under review, Wu et al. (2005) showed that a mutant Htz1 protein that is not recognized by the SWR-C can still copurify with other core histones, indicating that Htz1 can be deposited into chromatin in an SWR-C-independent manner. It will be interesting to determine whether an alternative deposition pathway exists and whether its activity is regulated by Htz1 acetylation.

Why would Htz1 acetylation and Htz1 deposition take place mainly at active promoters, as nucleosomes are lost? This may be explained when we consider that histones in euchromatin are likely to be in a dynamic equilibrium between "assembled" and "disassembled" states. In this model, the equilibrium is shifted toward histone disassembly at active genes, and toward assembly at repressed genes, but the process is always dynamic. Even at genes whose expression levels are extremely high, nucleosomes are never completely lost (Boeger et al. 2003; Schwabish and Struhl 2004), which may be a reflection of the activities of several histone deposition complexes including FACT and Spt6 (Kaplan et al. 2003; Schwabish and Struhl 2004). Similarly, as nucleosomes containing Htz1 are lost from active promoters during induction, Htz1 may be continuously assembled onto promoter nucleosomes. This assembly is likely to be regulated by acetylation of Htz1.

Materials and methods

Yeast strains and plasmids

Lists of yeast strains and plasmids used in this study are provided in Supplementary Tables 1 and 2. Gene disruption and tagging were performed using standard techniques (Longtine et al. 1998). A 1.7-kb HindIII–SalI fragment containing the *HTZ1* gene and sequence encoding three N-terminal HA tags was amplified from CMY108 genomic DNA using *Pfu* Turbo DNA polymerase (Stratagene) and cloned into the yeast centromeric CEN/ARS, *URA3* vector pRS416 (Sikorski and Hieter 1989). Point mutations were introduced into the *HTZ1* coding sequence using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) and verified by sequencing. For integration into the endogenous *HTZ1* locus, HA-htz1 clones carrying point mutations were subcloned into pFA6a-KanMX (Wach et al. 1994). Genomic DNA was sequenced to verify point mutations in the *HTZ1* gene. Primer sequences are available upon request.

Purification of Htz1 and mass spectrometry

Yeast core histones were purified from asynchronous culture as described (Edmondson et al. 1996) and separated by reverse-phase (RP) C4 HPLC (Zhang et al. 2004). The fraction corresponding to Htz1 was digested by trypsin and analyzed by LC/MS/MS either immediately or after treatment with deuterate (D_6) acetyl anhydrite, as described previously (Smith et al. 2003; Zhang et al. 2004).

Generation of antibodies, ELISAs, and affinity purification

Peptides corresponding to fragments of Htz1 and carrying N-terminal cysteine residues were synthesized by the BioPolymer Synthesis Center at Caltech (Pasadena, CA). The sequences of these peptides, corresponding to residues 113–131 of Hta1, residues 119–133 of Htz1, and Htz1 residues 11–21, where residue 14 is acetylated, are shown in Supplementary Figure S1A. Peptides were conjugated via their cysteine residues to ovalbumin and used to immunize male rabbits as described (Suka et al. 2001). For ELISAs, KLH-conjugated peptides were used to coat 96-well plates, and competitor peptides at different concentrations were added before incubation with antisera.

For immunoaffinity purification, peptides were conjugated to UltraLink Iodoacetyl Gel according to the manufacturer's instructions (Pierce). Antisera were filtered and diluted 10-fold with 10 mM Tris-HCl (pH 7.5) before application to affinity columns. After extensive washing, affinity-purified antibodies were eluted in 100 mM glycine (pH 2.5). Antibodies were immediately restored to neutral pH and then dialyzed against PBS/50% glycerol before storage at –20°C. Polyclonal antibodies to the C terminus of H3 (Gunjan and Verreault 2003) were raised in rabbit and tested by Taiko To.

ChIP

ChIP was performed essentially as previously described (Hecht et al. 1999) except that cultures were grown to an OD_{600} of 0.8 before cross-linking. LPY3430 and related strains were grown at 37°C for 8 h prior to cross-linking to inactivate Esa1-L317S. For testing of antibody specificities, immunoprecipitates were assayed for protein by Western blotting or for coprecipitated DNA by PCR. Antibody amounts were determined in initial titration experiments, and each ChIP was performed from at least three independent cultures. Sequences of primers used in PCR are available upon request. For time-course analysis of *PHO5* induction and repression, cells were grown in high-phosphate or phosphate-free media, as described in Schermer et al. (2005). *PHO5* primers are those described in Reinke and Horz (2003).

Genome-wide binding microarrays

Microarrays containing ~6900 IGR fragments and 6500 ORF fragments, covering the whole *S. cerevisiae* genome, were custom printed at the University Health Network Microarray Center, University of Toronto. Probe preparation, hybridization, and washing were performed as described (Robyr and Grunstein 2003). Microarrays were scanned using an Agilent DNA Microarray Scanner, and images were processed and features extracted using Imagene 6.0 software (BioDiscovery). Data were processed with the following manipulations: background correction, flagging and removal of low-quality spots, normalization of overall array intensity, and scaling of data sets to a mean of 1. Average binding values were calculated from at least three biological replicates and used for subsequent analyses. Correlations between replicate data sets were >0.85. Microarray data are available in the Supplemental Material. For comparison of data sets, we used Access (Microsoft); for statistical analyses, we used Excel (Microsoft).

Normalization of acetylation to bulk histone levels

Others have recognized that the occupancy of bulk histone is an important consideration in ensuring that the profile of a modification is represented accurately (Deckert and Struhl 2001; Reinke and Horz 2003; Liu et al. 2005; Pokholok et al. 2005; van Leeuwen and van Steensel 2005; Wiren et al. 2005), and we believe that this is even more critical for a variant histone whose distribution is less homogeneous than the major core histones. We have normalized our Htz1-K14 acetylation data by dividing by total Htz1 occupancy both for single-gene analysis and in microarray experiments, where we compare to transcriptional frequency. When using this normalization approach for microarray data, it should be noted that dividing any data set by bulk histone binding data introduces a bias toward a positive correlation with transcription because histones are lost from active genes. The extent of this bias can be determined by randomizing the acetylation data set prior to normalization to bulk histone binding. When this is done we find that the randomized data does correlate with transcription ($r = 0.31$) but to a lesser extent than the unrandomized data (ORFs, $r = 0.51$; IGRs = 0.37).

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