Conserved Histone Variant H2A.Z Protects Euchromatin from the Ectopic Spread of Silent Heterochromatin

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Summary

Boundary elements hinder the spread of heterochromatin, yet these sites do not fully account for the preservation of adjacent euchromatin. Histone variant H2A.Z (Htz1 in yeast) replaces conventional H2A in many nucleosomes. Microarray analysis revealed that HTZ1-activated genes cluster near telomeres. The reduced expression of most of these genes in htz1Δ cells was reversed by the deletion of SIR2 (sir2Δ) suggesting that H2A.Z antagonizes telomeric silencing. Other Htz1-activated genes flank the silent HMR mating-type locus. Their requirement for Htz1 can be bypassed by sir2Δ or by a deletion encompassing the silencing nucleation sites in HMR. In htz1Δ cells, Sir2 and Sir3 spread into flanking euchromatic regions, producing changes in histone H4 acetylation and H3 4-methylation indicative of ectopic heterochromatin formation. Htz1 is enriched in these euchromatic regions and acts synergistically with a boundary element to prevent the spread of heterochromatin. Thus, euchromatin and heterochromatin each contains components that antagonize switching to the opposite chromatin state.

Introduction

Eukaryotic genomes are organized into two distinct yet interconvertible states, euchromatin and heterochromatin. While originally termed for their cytological appearances, it has become clear that each chromatin state comprises a series of different structures and that each can be thought of more broadly as being associated with a set of distinct functional and biochemical properties (reviewed by Richards and Elgin, 2002). Heterochromatin spreads from nucleation sites (silencers) across chromosomal regions, consuming euchromatin in the process, and converting it into a transcriptionally silent state. Integral to this process are alterations in the patterns of posttranslational modifications of histone H3 and H4 N-terminal tails, which permit the binding of heterochromatin-specific histone binding proteins required for silencing (reviewed by Jenuwein and Allis, 2001; Moazed, 2001).

Since heterochromatin formation resembles a nucleation-polymerization process, crucial regulatory mechanisms must exist that prevent the silencing machinery from exhausting its euchromatic substrate. However, little is known about such inhibitory mechanisms. Deletion of DNA sequences that lie at the boundaries of heterochromatic regions can result in further spread of heterochromatin. However, the precise molecular mechanisms by which such boundary elements function have been elusive (reviewed by Bell et al., 2001; Oki and Kamakaka, 2002). Because deletion of some boundary elements results in only a limited ectopic spread of heterochromatin (Noma et al., 2001), there must be undiscovered mechanisms which negatively regulate the process.

In the budding yeast, S. cerevisiae, silencing of the silent mating-type cassettes HMR and HML by the Sir proteins (Sir1–4) involves changes in chromosome structure characteristic of heterochromatin formation; these include decreases in accessibility to enzymatic probes and acetylation levels of histone tails (Braunstein et al., 1993; Moazed, 2001; Singh and Klar, 1992). Similarly, telomeric regions are silenced via the action of Sir2-4 and are also rendered into a heterochromatin-like state (Aparicio et al., 1991; Braunstein et al., 1993; Gottschling, 1992; Gottschling et al., 1990). In both instances, the silent mating-type cassettes and telomeric regions Sir2-4 are intrinsic components of silent heterochromatin (Hecht et al., 1996; Strahl-Bolsinger et al., 1997). Supporting the notion that histone tail modifications are critical to the specification of chromatin type in this system, purified Sir2 displays an NAD-dependent histone deacetylase activity that is required for silencing (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000). Moreover, analysis of mutants suggests that deacetylation of acetyl-lysine 16 of histone H4, a preferred in vitro target of Sir2, is important for silencing (Imai et al., 2000; Johnson et al., 1990). Current evidence is consistent with a sequential assembly model for Sir heterochromatin in which Sir2-4 is recruited to sites where silencing is initiated via interactions with sequence-specific DNA binding proteins (Hoppe et al., 2002; Luo et al., 2002; Rusche et al., 2002). Deacetylation of the tails of nearby histones in adjacent nucleosomes may promote the binding of Sir3 and Sir4, which directly bind histone H3 and H4 N-terminal tails with a strong preference for the underacetylated form of the H4 tail (Carmen et al., 2002). It has been proposed that this allows local recruitment of additional Sir2 via a protein-protein interaction with Sir4. Repeated cycles of deacetylation, Sir3/Sir4 binding, and recruitment Sir2 offers a plausible mechanistic framework for understanding the propagation of Sir heterochromatin (Moazed, 2001).

As in other eukaryotes, chromatin boundary elements have been identified in S. cerevisiae, which flank the silenced loci HMR and HML; others have been identified near telomeres (Bi and Broach, 2001). One example is a tRNA gene that occurs to the right of HMR (Donze et al., 1999). Although it is known that transcription of this gene by RNA polymerase III is required for its boundary function (Donze and Kamakaka, 2001), the molecular mechanism by which it inhibits silencing is incompletely understood.

Histone H2A.Z is a universally conserved, intrinsic component of eucharyotic chromatin that replaces the conventional H2A protein in a significant fraction of
nucleosomes (reviewed by Redon et al., 2002). Previous studies have indicated a role for H2A.Z in transcriptional activation. For example, in the ciliate Tetrahymena thermophila H2A.Z is associated with the transcriptionally active macronucleus but not the quiescent micronucleus (Allis et al., 1980). Additionally, in Saccharomyces cerevisiae the deletion of HTZ1 results in a defective induction of the GAL1 and PHO5 genes in response to their respective inducing signals, particularly in the absence of the SWI/SNF ATP-dependent nucleosome remodeling complex (Santisteban et al., 2000). However, H2A.Z has also been shown to promote gene silencing at reporter genes integrated at HMR and at a telomeric locus, leading to the proposal that it functions both in gene activation and gene silencing (Dhillon and Kamakaka, 2000).

We have utilized whole-genome microarray hybridization to identify genes that require Htz1 for their normal expression. We find that Htz1-activated genes are highly enriched near telomeres and often cluster together in small chromosomal domains. We show that Htz1 functions to protect genes from Sir-dependent silencing at these telomeric locations and in regions flanking the HMR silent cassette. High-resolution chromatin immunoprecipitation experiments revealed that, in the absence of Htz1, Sir2 and Sir3 spread outside of their normal boundaries at HMR concomitant with the appearance of histone tail acetylation and methylation modification patterns indicative of heterochromatin formation. The euchromatic regions that flank HMR are enriched for Htz1, whereas the region within HMR is relatively depleted. Genetic tests indicate that Htz1 antagonizes heterochromatin spread as part of a pathway, which is separate from that of a characterized boundary element. Thus, Htz1 is an intrinsic component of euchromatin that functions to antagonize the formation of Sir heterochromatin via a boundary element-independent pathway.

Results

Genes Requiring Htz1 for Normal Expression

Are Enriched near Telomeres

To identify the genes regulated by HTZ1 in yeast, we compared global transcript levels in htz1Δ mutant cells to wild-type cells by hybridization of differentially labeled cDNAs to whole-genome yeast microarrays (DeRisi et al., 1997). Four replicate experiments were performed using independently derived cultures, and genes positively and negatively regulated by HTZ1 were identified using the significance analysis of microarrays (SAM) package (Tusher et al., 2001). This analysis yielded 214 genes that are significantly activated by HTZ1 and 107 that are repressed by HTZ1. Visual inspection of the list of HTZ1-activated genes revealed that many were near telomeres. As shown in Figure 1, Htz1-activated genes are highly enriched within 30–40 kb of chromosome ends. This enrichment is not due to an increase in overall gene density at telomeres: analysis of the fraction of genes activated by Htz1 in 10 kb windows from chromosome ends reveals that a significant portion of genes within approximately 30 kb of telomeres are activated by Htz1 (Table 1). For example, 30% of genes between 10–20 kb of telomeres are activated by Htz1 (Table 1). In contrast, genes greater than 60 kb from telomeres are significantly depleted for Htz1-activated genes (Table 1). We note that genes repressed by Htz1 appear to be randomly distributed throughout the genome (data not shown).

A second striking characteristic of the set of Htz1-activated genes is that they tend to occur in small clusters along the chromosome, termed HZADs for Htz1-activated domains (Supplemental Table S1 available at http://www.cell.com/cgi/content/full/112/5/725/DC1). Eighteen such clusters can be discerned that contain three or more Htz1-activated genes occurring within 15 kb of each other. Of the 18 HZADs, 15 occur within 35 kb of a telomere (and 14 within 20 kb). For example, HZAD 15, which occurs on the right arm of chromosome XIV, is a cluster of five adjacent genes (YNR071C, HXT17, YNR073C, YNR074C, and COS10), that lie between 4.3 and 12.8 kb from the telomere (Supplemental Table S1 available at above website). Of the 134 Htz1-activated genes that are not near telomeres, only 10 form clusters (Supplemental Table S1 available at above website; HZADs 1, 7, and 8).

Htz1 Protects a Set of Telomere-Proximal Genes

from Sir2-Dependent Silencing

One explanation for the enrichment of Htz1-activated genes near chromosome ends would be that Htz1 protects genes from Sir-dependent telomeric silencing. We term such an activity “anti-silencing.” This hypothesis predicts that the expression defect of telomeric proximal genes in htz1Δ cells should be reversed by the removal of the silencing machinery. To test this prediction, we used microarray hybridization to examine the global transcript levels of an htz1Δ sir2Δ double mutant. Analysis of the data revealed that of 81 Htz1-activated genes within 35 kb of telomeres, the gene expression defect of 46 (57%) genes in htz1Δ strains was significantly reversed by deletion of SIR2 (Figure 2A). Thus, for the majority of telomeric Htz1-activated genes, Htz1 indirectly or directly protects them from being silenced by Sir2. The defect in gene expression in htz1Δ cells for the remaining 35 genes either showed no effect upon deletion of SIR2 or displayed a more enhanced defect in gene expression in the htz1Δ sir2Δ double mutant (Figure 2B). We suspect that these genes are also anti-silenced by Htz1, but for a Sir2-independent repression system (see Discussion). We note that of the 134 non-telomeric Htz1-activated genes, the expression defect for a fraction (24%) can be partially reversed by deletion of SIR2; however, suppression is considerably weaker than that observed for the telomeric Htz1-activated genes (see http://madhanilab.ucsf.edu/public/htz1/).

While our microarray analysis best supports the conclusion that Htz1 protects genes from Sir-dependent silencing, a trivial explanation for these results might be that silencing factor levels are increased in htz1Δ mutants. Although the observation that silencing of an artificial reporter inserted adjacent to a telomere is reduced in an htz1Δ mutant (Dhillon and Kamakaka, 2000) makes this possibility unlikely, we examined the levels of an integrated HA-tagged version of SIR3 by immunoblotting and found no difference in wild-type versus htz1Δ cells (Supplemental Figure S1 available at http://
Figure 1. Chromosomal Distribution of Genes that Require HTZ1 for Normal Expression

Shown is a histogram of Htz1-activated genes identified by microarray analysis plotted as a function of their distance from a chromosomal end.

www.cell.com/cgi/content/full/112/5/725/DC1). Also, inspection of our microarray data revealed no increases in the mRNA levels for genes encoding known silencing factors such as SIR1–4, RAP1, ABF1, and ORC genes (see http://madhanilab.ucsf.edu/public/htz1).

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HTZ1-activated genes within HZAD 2 (Figure 3B). Suppression inspection of our microarray data revealed no increases in the mRNA levels for genes encoding known silencing of the defect of htz1/H9004 cells by hmr/H9004 is highly specific for these seven loci (Supplemental Figure S2 available at http://www.cell.com/cgi/content/full/112/5/725/DC1) indicating that the effects of the HMR deletion are specific to the region around HMR. It is notable that several of the HMR-proximal loci display repression by Sir2 in an HTZ1− genetic background (e.g., YCR106W in Figure 3B), suggesting that a locus can be partially protected from silencing by Htz1; that is, in some cases, Htz1 may reduce but not eliminate heterochromatin formation.

Table 1. Genes Requiring Htz1 for Normal Expression Are Enriched near Telomeres

<table>
<thead>
<tr>
<th>Interval</th>
<th>Fraction Requiring Htz1</th>
<th>( \chi^2 )</th>
<th>( p )</th>
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<td>0–10 kb</td>
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<td>102.3</td>
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<tr>
<td>10–20 kb</td>
<td>0.298</td>
<td>264.8</td>
<td>&lt;0.001</td>
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<td>3.8</td>
<td>NS</td>
</tr>
<tr>
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<td>&lt;0.001</td>
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As Sir2-mediated silencing involves the deacetylation of N-terminal tails of histones, we first compared the histone acetylation landscape of the regions cited above in wild-type and htz1Δ cells to determine if loss of HTZ1 produced the predicted reduction in acetylation levels. To do this, we performed ChIP analysis using an antibody specific for tetra-acetylated histone H4 (Ac-H4). Representative gels are shown in Figure 4. In agreement with recent data, we observe a relative paucity of Ac-
H4 within HMR compared to the flanking euchromatic regions (Rusche et al., 2002). At regions upstream of genes just outside of HMR and near the right telomere chromosome XIV, we observed from -6.5- (YCR095C) to 17.5- (YCR106W) fold increases in abundance of Ac-H4 relative to the levels within HMR in wild-type cells. In cells lacking HTZ1, the relative enrichments of Ac-H4 are significantly reduced at all Htz1-activated genes, supporting the view that Htz1 protects these genes from Sir-mediated silencing (Figure 5A and 5B).

Set1-mediated methylation of histone H3 on lysine 4 (K4Me-H3) has been shown to be enriched outside of telomeric heterochromatin in S. cerevisiae, consistent with evidence from other fungal and metazoan systems that K4-MeH3 is a marker for euchromatin (Bernstein et al., 2002). To investigate this euchromatic marker in anti-silenced regions we used ChIP to compare K4Me-H3 levels in wild-type and htz1/H9004 strains (see Experimental Procedures). In wild-type cells, we detected a pattern that is similar to that of Ac-H4, namely a striking paucity of K4Me-H3 within HMR and a 4- to 13-fold relative enrichment of K4Me-H3 at anti-silenced regions outside of HMR and near YNR074C on chromosome XIV (Figures 5C and 5D). Again as with Ac-H4, cells lacking HTZ1 have reduced K4Me-H3 at the promoters of every gene protected from silencing by Htz1 with the exception of the region upstream of YCR095C (Figures 5C and 5D).

Recent work indicates that K4Me-H3 is more highly enriched in ORFs over promoters (Bernstein et al., 2002). Because our analysis was restricted to intergenic regions, we may have therefore underestimated the differences in K4Me-H3 levels in wild-type compared with htz1/H9004 at YCR095C and elsewhere. Nevertheless, these data show that in cells lacking HTZ1, anti-silenced regions acquire modification patterns (reduced Ac-H4 and K4Me-H3) consistent with the formation of ectopic heterochromatin.

Ectopic Spread of Sir2 and Sir3 in Cells Lacking HTZ1
To determine if the ectopic Sir-dependent silencing and changes in histone modification patterns in htz1Δ mutants is caused by the ectopic spread of components of the Sir complex, we used ChIP to compare the patterns of Sir2 and Sir3 association in wild-type and htz1Δ strains. In HTZ1 strains, we observed large enrichments of both Sir3 (58- to 68-fold) and Sir2 (19- to 40-fold) within the silenced region of HMR (Figures 6A and 6C), and much reduced levels of Sir2 and Sir3 outside of the boundaries of HMR. In htz1Δ strains, we observed large enrichments of both Sir3 (58- to 68-fold) and Sir2 (19- to 40-fold) within the silenced region of HMR (Figures 6A and 6C), and much reduced levels of Sir2 and Sir3 outside of the boundaries of HMR. In htz1Δ cells, the relative enrichment of Sir3 is significantly increased at all anti-silenced regions both proximal to HMR and on chromosome XIV (Figures 6A and 6B). For example, the strongly anti-silenced genes GIT1 and YNR074C have average Sir3 ChIP association values of approximately 1.2 and 2.3 in wild-type cells and these values increase to 3.2 and 12.4, respectively in htz1Δ mutants (Figure 6A). Similar results were obtained for Sir2 (Figures 6C and 6D), with increases ranging from 1.2- to 3.4-fold observed in the HMR-proximal region and 4.7-fold at YNR074C. We conclude that Htz1 functions to prevent the Sir complex from spreading into the euchromatic regions that are adjacent to silenced regions.

Figure 2. Suppression of the htz1Δ Transcription Defect near Telomeres by Deletion of SIR2
(A) Telomeric genes whose defect in htz1Δ is significantly suppressed by sir2Δ. Color representation of gene expression values of genes within 35 kb of a chromosome end whose expression defect in htz1Δ cells is suppressed by deletion of SIR2. Four replicate experiments are shown for each genotype. Color scale is shown for log2 of the mutant:wild-type gene expression ratios.
(B) Telomeric genes whose defect in htz1Δ is not significantly suppressed by sir2Δ. Color representation of gene expression values of genes within 35 kb of a chromosome end whose expression defect in htz1Δ cells is not suppressed by deletion of SIR2. Four replicate experiments are shown for each genotype. Scale is as in (A).
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Figure 3. Geography of Anti-Silencing

(A) Diagram of two prominent anti-silenced domains. Shown is a region of chromosome III containing the \textit{HMR} silent mating-type cassette and HZAD 2 and a chromosomal region near the right telomere of XIV corresponding to part of HZAD 15. Depicted are the locations of open reading frames found in these regions. Those subject to anti-silencing by Htz1 are indicated with arrows. Also indicated are the locations and designations of PCR primers used in this study for ChIP analysis.

(B) Effect of deletion of \textit{SIR2} or \textit{HMR} on the expression of \textit{HMR}-proximal genes. Shown are the average expression ratios derived from microarray hybridization relative to wild-type of the indicated genotypes at genes near \textit{HMR} that require \textit{HTZ1} for normal expression. Plotted are means and standard deviations for four independent experiments performed on each genotype.

In addition to the increase in Sir association in euchromatic regions in cells lacking \textit{HTZ1}, we detected a statistically significant decrease of Sir3 association within \textit{HMR} in \textit{htz1}\textbackslash\textit{Δ} mutants (Figures 6A and 6B). Accordingly, \textit{HMR} acquired slightly more euchromatin-specific histone modification patterns in \textit{htz1}\textbackslash\textit{Δ} mutants, with increased levels of Ac-H4 and K4Me-H3 (Figures 5B and 5D). These results are consistent with the observation that an \textit{ADE2} reporter gene integrated at \textit{HMR} is mildly derepressed in \textit{htz1}\textbackslash\textit{Δ} mutants (Dhillon and Kamakaka, 2000).

\textbf{Htz1 Is Enriched in Euchromatic Regions Outside of \textit{HMR}}

In principle, Htz1 could perform its anti-silencing function in one of three different regions: (1) Htz1 could act at silenced regions to tether Sir proteins to heterochromatin, thus preventing them from spreading ectopically; (2) Htz1 may function as a component of boundary elements that block the spread of heterochromatin beyond their normal bounds; or (3) Htz1 could manifest its protective function at the anti-silenced genes themselves.
Figure 4. Quantitative PCR Analysis of Tetra-Ac-H4 ChIP Pellets
Quantitative PCR with $^{32}$P-dATP incorporation was performed on Ac-H4 ChIP pellets and electrophoresed on polyacrylamide gels (see Experimental Procedures). Shown are representative phosphorimager scans of reactions performed on immunoprecipitation (IP) and input whole-cell extract (WCE) DNA from paired wild-type and mutant strains. Primer pair names are indicated above the gels. The genomic positions of these primers are shown in Figure 3A.

Figure 5. ChIP Analysis of Euchromatic Markers in Wild-Type and $htz1\Delta$
(A and C) Average relative Ac-H4 (A) and K4Me-H3 (C) enrichments for wild-type and $htz1\Delta$ are shown for each primer set with its amplified region denoted as indicated in Figures 3A and 4. Standard error of the mean (SEM) error bars are shown.
(B and D) The degree of change of Ac-H4 or H3 Me-K4 enrichments in $htz1\Delta$ versus wild-type for each locus was determined by dividing the $htz1\Delta$ enrichment value of each locus by the correlating enrichment value from a paired wild-type experiment. Shown are average $htz1\Delta$/WT Ac-H4 (B) and H3 Me-K4 (D) ratios for each locus with SEM error bars plotted on logarithmic scales. Values that are less than one indicate there is less enrichment at that locus in $htz1\Delta$ compared to wild-type.
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Figure 6. ChIP Analysis of Sir2 and Sir3 Enrichment in Wild-Type and htz1Δ

(A and C) Average Sir3 (A) and Sir2 (C) enrichments for wild-type and htz1Δ are shown as for Figures 5A and 5C.

(B and D) The degree of change in Sir3 (B) and Sir2 (D) enrichments in htz1Δ versus wild-type for each locus was determined as for Figures 5B and 5D. Average htz1Δ/WT ratios are shown plotted on logarithmic scales with SEM error bars. Values that are greater than one indicate that there is more enrichment at that locus in htz1Δ compared with wild-type.

To help distinguish between these possibilities, we used ChIP to determine the distribution of Htz1 along chromosomes (see Experimental Procedures). We found that Htz1 is enriched at the promoters of anti-silenced genes relative to HMR and the known boundary element that flanks HMR on the right (Figure 7A). For example, the promoters of GIT1 and YNR074C contain amongst the most abundant levels of Htz1 (7-fold higher than within HMR) and these genes are strongly anti-silenced by Htz1. While we cannot exclude the possibility that Htz1 is present at HMR and/or the boundary element at low levels and functions there, the enrichment of Htz1 at the promoters of genes protected from silencing by Htz1 is most consistent with it functioning in anti-silencing directly at these regions.

Because Htz1 has previously been shown to be present at the promoters of the non-telomeric genes PHO5 and GAL1 (Santisteban et al., 2000), we asked whether Htz1 is particularly enriched in euchromatic regions flanking heterochromatin or whether the levels of Htz1 in these anti-silenced regions are comparable to its levels at other euchromatic regions. We used ChIP to examine the levels of Htz1 at several well-characterized loci that are distant to telomeres (GPD1, CYC1, GAL1, PHO5, ADH1, and ACT1) and compared them to levels found at HMR (primer D) and GIT1 (Supplemental Figure S3 available at http://www.cell.com/cgi/content/full/112/5/725/DC1). In general we found that Htz1 was enriched at these regions relative to HMR, though not as much so as at GIT1. Htz1 was shown previously to be enriched in the promoters versus coding sequences of the GAL1 and PHO5 genes, consistent with a role in transcriptional initiation (Santisteban et al., 2000). We confirmed that Htz1 is more enriched in the promoters of GAL1 and PHO5 compared with their respective ORFs and observed a similar pattern for GPD1. Interestingly however, for CYC1 at least as much Htz1 was present in the ORF as at the promoter and for ADH1 and ACT1, Htz1 was more abundant in the respective ORF regions than the promoters. We also examined the levels of Htz1 in two additional heterochromatic regions, the protosilencers contained within the repetitive subtelomeric elements core X and Y and found that, like within HMR, Htz1 is depleted from these regions (Supplemental Figure S3...
characterized boundary element (BE) that flanks the right side of HMR. If HTZ1 and BE function in separate anti-silencing pathways, then the htz1Δ beΔ double mutant should display a more severe phenotype than either htz1Δ or beΔ single mutant. We constructed isogenic strains of the following genotypes: beΔ, htz1Δ, beΔ htz1Δ, or wild-type for these two loci. We then used ChIP with antibodies to Sir3 to examine the ectopic spread of Sir3 in these strains. We focused our analysis on GIT1, the most proximal gene to the HMR right boundary element. As shown in Figure 7B, both beΔ and htz1Δ strains show an increase in the association of Sir3 at GIT1 relative to wild-type (~2-fold increase). The htz1Δ beΔ double mutant shows a significant further increase in Sir3 association at GIT1 compared with either of the single mutants (~4-fold increase compared with WT), consistent with Htz1 acting independently of this boundary element to antagonize the ectopic spread of Sir3 to the GIT1 locus.

Discussion

Recent studies have begun to reveal the mechanisms by which silent heterochromatin is initiated and propagated. In both yeast and mammals, silencing initiates at a specific site and then spreads across a chromosome converting euchromatin into heterochromatin via alterations in histone modification states that are in turn recognized by proteins required for heterochromatin formation (reviewed by Jenuwein and Allis, 2001; Moazed, 2001). For instance, during mammalian X chromosome inactivation, silencing and heterochromatin formation initiates at the X-inactivation center and spreads outward, coating the chromosome with the Xist non-coding RNA, which is itself required for the initiation and maintenance of the process (reviewed by Plath et al., 2002). Likewise, in yeast, silencing at the HMR silent mating-type cassette initiates at the E and I silencers and spreads, coating that chromosomal region with the Sir complex (reviewed by Moazed, 2001). These observations have led to the general concept that euchromatin can be converted to the heterochromatic state through the action of a specialized silencing machinery.

H2A.Z Antagonizes Sir-Dependent Silencing and Heterochromatin Formation

In this paper, we have described evidence that in budding yeast, the universally conserved histone variant H2A.Z functions to antagonize Sir-dependent gene silencing and heterochromatin formation. Global gene expression measurements revealed that genes located near chromosomal domains subject to silencing, namely telomeres and the silent mating type cassette HMR, require Htz1 for their full expression. For the majority of these genes, the defect in gene expression in htz1Δ cells can be reversed by deletion of SIR2, indicating that H2A.Z inhibits Sir2-dependent silencing. Supporting this conclusion, the gene expression defects in htz1Δ cells of the genes flanking HMR were specifically reversed by a deletion that removes both the E and I silencers of HMR, which are the nucleation sites for the formation of Sir heterochromatin at this site. Our observation that some telomeric genes require HTZ1 for a reason unre-
lated to Sir-dependent silencing may be explained by a second type of Sir-like telomeric repression recently described that is mediated by the Hda1 histone deacetylase together with the Tup1/Ssn6 corepressor rather than by the Sir complex (Robyr et al., 2002). Indeed, several of the HZADs (HZAD 5, 12, 13, 16–18) contain genes that occur within previously reported Hda1-repressed (HAST) domains (Supplemental Table S1 available at http://www.cell.com/cgi/content/full/112/5/725/DC1). Thus, Htz1 may function to protect some telomeric genes from Hda1-dependent or at an as yet undiscovered mode of Sir-independent telomeric repression (Wyrick et al., 1999).

Using chromatin immunoprecipitation, we observed the ectopic spread of Sir3 and Sir2 in htz1Δ mutants beyond their normal boundaries at HMR. These data are in agreement with our microarray-based genetic suppression experiments, which indicate that Htz1 protects these genes from Sir-mediated silencing. Our results show that Htz1 acts to inhibit silencing upstream of the physical spread of the Sir complex as opposed to a step that occurs after the association of the Sir complex. We also identified a similar increase in Sir protein association near the right telomere of chromosome XIV, particularly at the YNR074C locus, which is strongly dependent on HTZ1 for its expression. Concomitant with the spread of Sir proteins in htz1Δ cells, ChIP analysis revealed a decrease in both acetylated histone H4 and lysine 4-methylated histone H3 at anti-silenced loci (Figures 4 and 5). Methyl-lysine 4 of H3 has been proposed as a euchromatin-specific “mark” in other eukaryotes, but whether it serves this purpose in S. cerevisiae has been controversial (Braunstein et al., 1993; Briggs et al., 2001; Bryk et al., 2002; Nislow et al., 1997; Turner, 2002). Our observation that K4-methylated H3 is enriched in the euchromatic regions outside of HMR is more consistent with the view that this modification is a conserved marker for euchromatin (Bernstein et al., 2002). Moreover, an association between H3 K4 trimethylation and gene activity has recently been reported (Santos-Rosa et al., 2002).

While our global microarray and ChIP analyses show that Htz1 antagonizes silencing, a previous study concluded that Htz1 promotes silencing. This conclusion was based largely on the observations that the silencing of an ADE2 reporter gene integrated within HMR and a UR43 reporter integrated adjacent to a telomere show decreased silencing in htz1Δ cells and that the overexpression of HTZ1 was found to suppress the silencing defect of some hypomorphic alleles of SIR1 (Dhillon and Kamakaka, 2000). One way to reconcile these observations relies on the finding that Sir protein pools are limiting for silencing (Maillet et al., 1996; Smith et al., 1998). We observe a small but significant decrease in Sir3 association and correlating increases in euchromatic histone modifications within HMR in htz1Δ mutants, suggesting that association of Sir with ectopic sites in htz1Δ mutants results in a depletion of the pool available to nucleate silencing elsewhere. The observed decreases in silencing of the two aforementioned reporter genes in htz1Δ could thus be due to a redistribution of silencing factors. Likewise, the ability of HTZ1 overexpression to suppress sir1 mutant alleles could reflect increased availability of Sir proteins caused by improved anti-silencing outside of HMR and elsewhere. Other factors previously thought to be involved in promoting silencing might actually be anti-silencing factors whose loss results in a redistribution of Sir proteins to sites. In particular, our observation that K4-MeH3 is highly enriched in euchromatic regions flanking heterochromatin suggests that the previously described defects in silencing observed in cells lacking the H3 4-methylase Set1 may be indirect.

To summarize, Sir proteins spread beyond their normal bounds in htz1Δ cells and this spread is accompanied by ectopic transcriptional silencing that is accompanied by the formation of chromatin with a heterochromatine-like histone tail modification pattern. While the simplest model is that Sir proteins spread along the chromosome in htz1Δ cells, we cannot rule out the possibility that Htz1 functions by preventing the activation of unidentified cryptic protosilencers similar to those identified in the subtelomeric elements core X and Y′ (reviewed by Fourel et al., 2002). We note that our examination of Htz1 levels at core X and Y′ corresponded to the sites of previously defined protosilencers; however, no enrichment of Htz1 at these sites was apparent making it less likely that it acts there (Supplemental Figure S2 available at http://www.cell.com/cgi/content/full/112/5/725/DC1).

How Does Htz1 Mediate Anti-Silencing?

To begin to determine how Htz1 mediates anti-silencing, we first determined the relative enrichments of Htz1 at silenced regions compared with the anti-silenced regions (Figure 7A and Supplemental Figure S2 available at above website). We observed a relative enrichment of Htz1 at anti-silenced genes and a paucity of Htz1 within silenced regions. This observation supports the model that Htz1 acts directly at anti-silenced genes to protect them from silencing that is initiated at nearby silencers. We also addressed the possibility that Htz1 contributes to the function of a known boundary element, which flanks HMR (BE). Our results indicate that BE and HTZ1 define separate parallel mechanisms for antagonizing the spread of silencing: (1) Htz1 is relatively depleted at BE (Figure 7A), and (2) beΔ htz1Δ double mutants exhibit synergistic anti-silencing defects (Figure 7B). As the spread of heterochromatin across chromosomes seems likely to be a highly cooperative process, it may have been necessary for cells to evolve multiple redundant mechanisms to antagonize its consumption of euchromatin.

The anti-silencing activity we have described for Htz1 may be an intrinsic property of chromatin harboring this molecule: studies of purified nucleosome arrays containing H2A.Z versus H2A have found that H2A.Z-containing chromatin is inherently resistant to condensation, at least in vitro (Abbott et al., 2001; Fan et al., 2002). Being an integral component of nucleosomes, Htz1 may thus antagonize silencing due to its effects on the biophysical properties of chromatin. That is, Htz1 nucleosomes may be refractory to the mechanisms by which the Sir complex spreads through and condenses chromatin. Ultimately, high-resolution structural analysis of silent chromatin may yield insights into what aspect of heterochromatin formation is disrupted by H2A substitution by H2A.Z.
Recently, Dot1 has been identified as a histone H3 lysine 79 methylase (Lacoste et al., 2002; Ng et al., 2002; van Leeuwen et al., 2002). It has been proposed that methylation at this site prevents the binding of Sir proteins and thereby serves to antagonize silencing (van Leeuwen et al., 2002). Others have proposed that this modification is required to tether Sir proteins at telomeric silenced sites to promote silencing (Ng et al., 2002). The observation that the fraction of H3 that is methylated (90%) is considerably larger than the small fraction of the genome that is near telomeres makes this second explanation less likely (van Leeuwen et al., 2002). Examination of the distribution of H3-K79 methylation across defined heterochromatin-euchromatin junctions, such as those that flank HMR may help to resolve this issue. In any case, comparison of the gene expression defects seen in dot1Δ cells (Hughes et al., 2000) to those of htz1Δ cells reveals no significant overlap (data not shown), suggesting that regardless of the precise function of H3-K79 methylation, it differs from that of Htz1.

Very recent observations indicate that the histone acetylase homolog Sas2 antagonizes silencing near telomeres in yeast by promoting the acetylation of lysine 16 of histone H4 (Kimura et al., 2002; Suka et al., 2002). Interestingly, sas2Δ and htz1Δ mutants show significant similarities in their gene expression defects (M.W. and H.D.M., unpublished data) suggesting that Htz1 and Sas2 may define a multi-step anti-silencing pathway. An intriguing possibility is that acetylation of lysine 16 directs the deposition of H2A.Z in euchromatin.

The SWI/SNF ATP-dependent remodeling complex and Htz1 function redundantly in the activation of the GAL1 and PHO5 genes, two well-studied inducible genes (Santisteban et al., 2000). This study indicated that, although Htz1 is required for the induction of these two genes, it associates with these promoters in the repressed state prior to induction and appears to be partially displaced from the genes upon induction (Santisteban et al., 2000). This novel behavior distinguishes Htz1 from conventional coactivators such as the SAGA complex, which is recruited by acetyl activators in the course of transcriptional induction (Bhaumik and Green, 2001; Larschan and Winston, 2001). These observations suggest that Htz1 is required very early in the pathway of transcriptional activation, consistent with the view that it functions to promote transcriptional competence prior to the action of conventional activators. Since transcriptional competence is a hallmark of euchromatin, the function of Htz1 in gene induction and in anti-silencing may be highly related or even the same: to antagonize the formation of condensed chromatin. Intriguingly, the SWI/SNF complex is particularly required for the expression of genes during mitosis, when chromatin is more condensed (Krebs et al., 2000). Similarly, the requirement for this complex in htz1Δ for the induction of the GAL1 and PHO5 genes may reflect a more condensed, heterochromatin-like state of these loci in htz1Δ cells. Consistent with this model, accessibility of the PHO5 promoter to restriction endonuclease digestion is reduced in htz1Δ cells (Santisteban et al., 2000).

Thus, while our studies indicate a critical role for Htz1 for the expression of genes near silenced domains, the association with Htz1 with the promoters of GAL1 and PHO5 (neither lie near characterized silenced regions) suggests that Htz1 may have a broader, albeit redundant role in gene expression. Such a view would be consistent with our finding that Htz1 is enriched at many euchromatic loci. Alternatively, it could be that the primary function of Htz1 is to protect euchromatic genes from silencing, but that this effect is only apparent under standard laboratory conditions for genes near silent domains.

Is the Relationship between Euchromatin and Anti-Silencing Analogous to that between Heterochromatin and Silencing?

We have described a conserved, euchromatin-enriched protein, Htz1, that antagonizes the formation of the opposite chromatin state. Conceptually, Htz1 can be viewed as a euchromatic counterpart to a heterochromatic silencing protein that coats heterochromatin and promotes its formation. Euchromatin may not be merely a state of chromatin that is consumed by specialized silencing factors; rather, the situation may be more symmetric with each type of chromatin harboring factors that antagonize the switch to the opposite chromatin state. This view raises the speculative question of whether there exist mechanisms by which euchromatin formation is an active process that initiates at specific sites and spreads at the expense of heterochromatin to determine the epigenetic landscape of the genome.

Experimental Procedures

Yeast Strains

Strains used in this study are described in Supplemental Table S2 available at http://www.cell.com/cgi/content/full/112/5/725/DC1.

Microarray Hybridization Procedures

Exponentially growing cells in YPD media (Q-Biogene) supplemented with tryptophan and adenine were diluted to an O.D. 600 of 0.15 and grown to an O.D. of 0.7 at 30°C. For each mutant culture, a paired wild-type culture was grown in an adjacent slot in a floor shaker. Cultures were rapidly harvested by vacuum filtration onto a Millipore nitrocellulose filter, which was then immediately placed in a 15 ml polypropylene tube and flash-frozen in liquid nitrogen. Total RNA was isolated as described (www.microarrays.org) and mRNA was selected using biotinylated oligo-dT and magnetic streptavidin beads (PromeGa) using the manufacturer’s protocol.

Microarrays were constructed as described (DeRisi et al., 1997). Yeast ORFs were amplified using a commercially available primers and yeast genomic DNA as a template (Research Genetics). Products were analyzed by gel electrophoresis, precipitated, resuspended in 3× SSC, and spotted onto poly-lysine coated microcroscope slides using a robot custom-built by Dr. Joseph DeRisi (UCSF). Detailed protocols are available at www.microarrays.org.

Cy3 and Cy5 labeled cDNAs were generated and hybridized to arrays for 6 hr at 65°C. For each experiment, four replicate experiments were performed, and the Cy3 and Cy5 labeling scheme was alternated to allow the subsequent removal of systematic biases arising from the dyes from the data by averaging. After washing and drying, arrays were scanned using a GenPix 4000 scanner (Axon, Inc.). Following spot gridding and data extraction, the data were uploaded on an AMAD database (www.microarrays.org) running on an Intel-based PC.

Microarray Data Analysis

Genes significantly different from wild-type in htz1Δ cells were identified using the significance analysis of microarrays (SAM) package (Tusher et al., 2001). Missing values were estimated using the nearest 10 neighbors. Imputed datasets were analyzed using delta fac-
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tors that yielded approximately 10% estimated false positives. This was found empirically to be a good trade-off between sensitivity (false negatives) and specificity (false positives), and yielded results similar to those obtained using arbitrary cut-offs to identify regulated genes. SAM analysis was also used to identify genes whose defect in htz1Δ could be reversed by deletion of Sir2 or HMR. Htz1-activated domains (HZADs) were defined by three or more Htz1-activated genes occurring within a 15 kb interval (Supplemental Table S1 available at http://www.cell.com/cgi/content/full/112/5/725/DC1). The full data set is available at http://madhanilab.ucsf.edu/public/htz1/.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed essentially as described (Strahl-Bolsinger et al., 1997). For Htz1 ChIP, YM1730 (htz1Δ) was transformed with a plasmid that expresses an N terminally triple-HA tagged functional version of Htz1. Crosslinking times for the Htz1 ChIP were 60 min. For all other experiments, crosslinking times were 15 min. Pellet and whole-cell extract DNAs were analyzed by quantitative PCR performed in the linear range using 32P-dATP incorporation, electrophoresis through 6% polyacrylamide gels in Tris-Borate-EDTA buffer, and phosphorimagery quantitation of radioactive bands in the dried gels. Enrichments relative to the PRP8 internal control were calculated as follows using the method of Noma et al. (2001). We normalized the enrichment values by using multiplex PCR and determined abundance ratios for each locus relative to a control PCR product corresponding to the middle of the open reading of PRP8, a gene for which there is no HTZ1-mediated regulation (our unpublished data). The pellet ratios were in turn normalized for amplification bias after division by the corresponding input whole-cell extract ratio to yield a value, which reflects relative abundance. For experiments shown in Supplemental Figure S2 (available at http://www.cell.com/cgi/content/full/112/5/725/DC1), quantitative PCR was performed using real-time PCR with SIBR green as a label. All of our ChIP experiments were performed in triplicate on paired wild-type and htz1Δ mutant strains where appropriate.

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