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Type B Histone Acetyltransferase Hat1p Participates in Telomeric Silencing

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Hat1p and Hat2p are the two subunits of a type B histone acetyltransferase from *Saccharomyces cerevisiae* that acetylates free histone H4 on lysine 12 in vitro. However, the role for these gene products in chromatin function has been unclear, as deletions of the *HAT1* and/or *HAT2* gene displayed no obvious phenotype. We have now identified a role for Hat1p and Hat2p in telomeric silencing. Telomeric silencing is the transcriptional repression of telomere-proximal genes and is mediated by a special chromatin structure. While there was no change in the level of silencing on a telomeric gene when the *HAT1* or *HAT2* gene was deleted, a significant silencing defect was observed when *hat1*Δ or *hat2*Δ was combined with mutations of the histone H3 NH_2-terminal tail. Specifically, when at least two lysine residues were changed to arginine in the histone H3 tail, a *hat1*Δ-dependent telomeric silencing defect was observed. The most dramatic effects were seen when one of the two changes was in lysine 14. In further analysis, we found that a single lysine out of the five in the histone H3 tail was sufficient to mediate silencing. However, K14 was the best at preserving silencing, followed by K23 and then K27; K9 and K18 alone were insufficient. Mutational analysis of the histone H4 tail indicated that the role of Hat1p in telomeric silencing was mediated solely through lysine 12. Thus, in contrast to other histone acetyltransferases, Hat1p activity was required for transcriptional repression rather than gene activation.

The DNA of eukaryotic cells is packaged in a nucleoprotein complex known as chromatin. The fundamental unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped around a protein core of histones H2A, H2B, H3, and H4. The core histones are important not only for the structural packaging of DNA in the nucleus but also for regulating many cellular processes that use DNA as a substrate. Much of the regulatory potential of the histones lies in their NH_2 termini. The NH_2-terminal tails, the first ~30 amino acids of each histone, are largely unstructured and contain high concentrations of lysine and arginine residues (37). The physical characteristics of the NH_2-terminal tails are regulated by extensive post-translational modifications, which include phosphorylation, methylation, ubiquitination, ADP-ribosylation, and acetylation (69).

Acetylation of core histone NH_2-terminal tails was discovered more than 30 years ago and has been the most extensively studied histone modification (3). Histone acetylation occurs on lysine residues, neutralizing their positive charge and changing their structure. As such, this modification is likely to affect the interaction of histones with both DNA and other proteins. The acetylation of the core histones is a dynamic process, with the acetylation state of a given histone determined by the actions of enzymes that add acetyl groups (histone acetyltransferases) and enzymes that remove them (histone deacetylases) (20, 27).

Histone acetyltransferases have traditionally been divided into two types, A and B. Type A histone acetyltransferases are nuclear enzymes that acetylate histones in a chromatin context. It was originally proposed that these enzymes were responsible for the increased levels of histone acetylation that are correlated with transcriptional activation. There is ample experimental evidence to support this idea, beginning with the discovery that the *GCN5* gene product of *Saccharomyces cerevisiae*, a transcriptional coactivator of several genes, is a histone acetyltransferase (12). A number of other proteins known to be involved in transcriptional regulation have also been identified as type A histone acetyltransferases, including P/CAF, CBP, P300, $TAF_{II}250$, ACTR, SRC-1, Tip60, Esa1p, and Elp3 (14, 16, 41, 44, 58, 61, 72–74). Thus, the acetylation of histones plays an important role in the activation of transcription.

Type B histone acetyltransferases are cytoplasmic enzymes that acetylate histones not associated with DNA. These enzymes are thought to be involved in the acetylation of newly synthesized histones (primarily H3 and H4) (11). When histone H4 is synthesized, it is rapidly acetylated in a specific, evolutionarily conserved pattern (52). There are four lysine residues in the histone H4 NH_2 terminus, located at positions 5, 8, 12, and 16. In all eukaryotes that have been examined, newly synthesized histone H4 is acetylated at positions 5 and 12, with little or no acetylation seen at positions 8 and 16 (15, 60). It should be noted that the acetylation state of newly synthesized histone H4 in yeast has not been determined. In many organisms, newly synthesized histone H3 is also acetylated, but not in a strictly conserved pattern (33, 60).

It seems likely that acetylation of histones H3 and H4 plays an early role in chromatin assembly. Pulse-chase experiments indicate that this acetylation occurs rapidly after the synthesis of the histones (33, 52, 60). In addition, diacetylated histone H4 is complexed with histone H3 in the cytoplasm (13). Two protein complexes, chromatin assembly factor 1 (CAF-1) and replication-coupling assembly factor (RCAF), mediate replication-dependent chromatin assembly in vitro. Both of these activities preferentially interact with H3-H4 tetramers that contain species of H4 that are acetylated in patterns similar to those of the newly synthesized protein (30, 59, 68, 71). The population of histone H4 that associates with CAF-1 is acetylated at lysines 5, 8, and 12, while that associated with RCAF

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is acetylated at only lysines 5 and 12 (30, 68, 71). CAF-1 and RCAF have homologs in *S. cerevisiae* (*CAC1*, *CAC2*, and *MSI1* for CAF-1 and *ASF1* for RCAF), and genetic analyses suggest that these complexes are both involved in some aspect of chromatin assembly in vivo (18, 31, 34, 42, 56, 68).

The Hat1p-Hat2p complex isolated from *S. cerevisiae* is the quintessential type B histone acetyltransferase (26, 32, 46, 70). Hat1p, the catalytic subunit of the enzyme, when expressed in bacteria, acetylates histone H4 at the same residues that are modified on newly synthesized histone H4, lysines 5 and 12 (32, 46). However, its activity in vivo may be restricted, as the native enzyme acetylates H4 only at lysine 12 (46). Hat2p is a regulatory subunit of the enzyme; it is not required for catalytic activity but increases specific activity 10-fold. Hat2p appears to function by mediating the interaction between Hat1p and histone H4 (46, 70). Hat2p is an ortholog of two nearly identical human proteins, Rbap48 and Rbap46 (47, 48). Proteins in the Hat2p/Rbap48 family are subunits of protein complexes that modulate chromatin structure, including CAF-1, the nucleosome remodeling factor, and several histone deacetylase and transcriptional corepressor complexes (24, 40, 64, 71, 75). Thus, these proteins seem to play a central role in the communication between histones and chromatin-modifying activities.

Simple genetic analysis of *HAT1* did not uncover an obvious role for type B histone acetyltransferases. Deletion of the gene does not affect cell growth or result in any other observable phenotype (32, 46). The lack of a *hat1*Δ phenotype suggested that type B histone acetyltransferases are not essential, but conservation of the acetylation pattern of newly synthesized histone H4 and of the Hat1p-Hat2p enzyme over a wide range of eukaryotes argues that this histone modification is important (15, 26, 60, 70). The lack of a phenotype may be explained by an acetyltransferase activity that can modify histone H4 just as Hat1p does or by structural redundancy in the histones that obviate the acetylation of histone H4 by Hat1p (35, 38).

In an effort to identify an in vivo role for the Hat1p-Hat2p histone acetyltransferase, we sought a sensitive genetic assay that monitored subtle changes in chromatin structure. Specifically, we explored whether Hat1p-Hat2p might play a role in telomeric silencing. Genes located near telomeres are transcriptionally repressed, or silenced, due to a special chromatin structure that is assembled over telomere-proximal DNA (19). Critical components of telomeric silent chromatin include the SIR proteins (Sir2p, Sir3p, and Sir4p), Rap1p (a telomere DNA-binding protein), and the histone H3 and H4 tails. It appears that the Sir proteins are recruited to telomeres through their interactions with Rap1p and one another and then "polymerize" along telomere-adjacent DNA by binding the NH_2-terminal tails of histone H3 and H4 of the associated nucleosomes (4, 22, 51, 55). Telomeric silent chromatin shares many of the hallmarks of heterochromatin in other eukaryotes, including a distinct acetylation pattern on the histone H4 NH_2-terminal tail. Histone H4 found in yeast silent chromatin is almost entirely unacetylated at positions 5, 8, and 16 but highly acetylated at position 12, just as it is in heterochromatin (9, 10). While it is not known whether this acetylation pattern plays a role in the formation of silent chromatin, the fact that Hat1p can specifically acetylate histone H4 at position 12 suggests that Hat1p may be necessary for silencing.

MATERIALS AND METHODS

Plasmid construction. A plasmid containing wild-type *HHT2* and *HHF2* was constructed from pRM200 (39). First, pRM200 was digested with *Eco*RI and *Xba*I. The 4.6-kbp fragment containing *HHT2* and *HHF2* was isolated and blunt-end ligated into the *Hin*cII site of pUC9 to generate pMP1. Then 1.4 kbp of *HHT2* downstream sequence was removed from pMP1 by digestion with *Hin*cII and *Sal*I, followed by blunt-end religation (pMP2). The 2.7-kbp *Pst*I fragment of pMP2 was ligated into the *Pst*I site of pRS314 to generate pMP3, which was used as the wild-type *HHT2-HHF2* plasmid in these studies.

A second plasmid containing the same fragment of *HHT2* and *HHF2* but with *HHT2* lysines 9, 14, 18, and 23 changed to arginines (K9,14,18,23R) was constructed by ligating the 2.7-kbp *Pst*I fragment of pRM253 into the *Pst*I site of pRS314 (pMP6) (39). The H3 K9,14,18,23,27R allele was constructed from pMP6 by PCR amplification of a 1.4-kbp fragment extending from the *Nco*I site downstream of *HHF2* to the *Pin*AI site in the *HHT2* open reading frame using the following primers: *HHF2 Nco*I, GGATTCCATGGGTTTCTGCG, and H3 K27R, CACCACCGGTAGATGGGGCGGATCTTCTGGCAGCTCTGGAG GC. The H3 K27R primers incorporate a mutation that changes HHT2 lysine 27 to arginine. This PCR fragment was digested with *Nco*I and *Pin*AI and ligated into pMP6 that had been digested with the same enzymes to generate pMP8. The *HHT2* K27R allele was confirmed by DNA sequencing.

All other *HHT2* and *HHF2* alleles were generated incrementally from pMP3, pMP6, and pMP8 by site-directed mutagenesis (Quik-Change site-directed mutagenesis kit; Stratagene) using appropriate combinations of the following PCR primers: H3 R9K A, CTAAACAAACAGCTAGAAAATCCACTGGTGG; H3 R9K B, CCACCAGTGGATTTTCTAGCTGTTTGTTTAG; H3 R14K A, CCA CTGGTGGTAAAGCCCCAAGAA; H3 R14K B, TTCTTGGGGCTTTACCA CCAGTGG; H3 R18K A, GCCCCAAGAAAACAATTAGCC; H3 R18K B, GGCTAATTGTTTTCTTGGGGC; H3 R23K A, CAATTAGCCTCCAAAGC TGCCAGAA; H3 R23K B, TTCTGGCAGCTTTGGAGGCTAATT; H4 K5R A, GCCTGGTAGAGGTAGAGGTGGTAAAGGTCTAGG; H4 K5R B, CCT AGACCTTTACCACCTCTACCTCTACCTGGC; H4 K8R A, GAGGTAAAG GTGGTAGAGGTCTAGGAAAAGG; H4 K8R B, CCTTTTCTCAGACCTCT ACCACCTTGACCTC; H4 K12R A, GGTCTAGGAAGAGGTGGTGCC; H4 K12R B, GGCACCACCTCTTCCTAGACC; H4 K16R A, GGAAAAGGTGG TGCCAGACGTCACAGAAAGATTC; and H4 K16R B, GAATCTTTCTGT GACGTCTGGCACCACCTTTTCC.

Following site-directed mutagenesis, the coding sequences were checked by DNA sequencing to confirm that no PCR artifacts were incorporated into the plasmids.

The 2.7-kbp *Pst*I fragment from pMP2 was ligated into the *Pst*I site of pRS317 to generate pMP9. The same *Pst*I fragment was also inserted into the *Pst*I site of pRS412 to generate pRS412/HHT2-HHF2.

pHAT1::LYS2 was made by first deleting the *Bam*HI-*Bgl*II fragment from the *HAT1* open reading frame in pT7Sc-HAT1 (46). A *Sal*I-*Sma*I fragment from YDpK containing the *LYS2* coding sequence was then blunt-end ligated into the *Bam*HI and *Bgl*II sites in pT7Sc-HAT1 (6).

Yeast strain construction. Standard yeast culture and genetic methods were used (1, 23). Gene deletions were confirmed by Southern blotting or colony PCR.

Strain UCC1111 [*MAT*α *ade2*::*his3*Δ*200 leu2*Δ*0 lys2*Δ*0 met15*Δ*0 trp1*Δ*63 ura3*Δ*0 adh4*::*URA3*-TEL (VII-L) *hhf2-hht2*::*MET15 hhf1-hht1*::*LEU2* pRS412 (*ADE2 CEN ARS*) - *HHF2-HHT2*] was constructed as follows. *URA3* was placed at the left arm of chromosome VII in strain BY4705 as described previously to generate UCC1091 (8, 19). The *HHT2-HHF2* gene pair was replaced by *MET15* using PCR-mediated gene disruption (UCC1095) (5). pMP9 was transformed into UCC1095, followed by replacement of the *HHT1-HHF1* gene pair with *HIS3* (UCC1098). The *LEU2* gene was then inserted in place of the *HIS3* gene by PCR-mediated gene disruption. pRS412-*HHT2-HHF2* was then swapped with pMP9 to generate UCC1111.

The *HAT1* and *HAT2* genes were each disrupted in UCC1111 with *HIS3* using PCR-mediated gene disruption to generate strains MPY1 and TKY101, respectively. *HAT1* was disrupted with *LYS2* in TKY101 by transformation with plasmid pHAT1::*LYS2* that had been digested with *Eco*RI and *Hae*II to generate TKY104.

Plasmids containing wild-type or mutant *HHT2-HHF2* alleles were transformed into these strains and selected on plates lacking tryptophan. Colonies that had lost the pRS412-*HHT2-HHF2* plasmid, and which were thus *ade2*, were identified by their red color.

BY4705a (*MAT***a** *ade2*::*his*Δ*200 leu2*Δ*0 lys2*Δ*0 met15*Δ*0 trp1*Δ*63 ura3*Δ*0*) was produced by changing the mating type of BY4705 by two-step replacement.

UCC6580 [*MAT*α *ade2*::*hisG his3*Δ*200 leu2*Δ*0 lys2*Δ*0 met15*Δ*0 trp1*Δ*63 ura3*Δ*0 adh4*::*URA3*-TEL (VII-L) *sir3*::*HIS3*] was generated by PCR-mediated disruption of the *SIR3* gene in UCC1091.

Telomeric silencing assays. Telomeric silencing was assayed essentially as described previously (19). Briefly, individual colonies of the indicated strains were resuspended in 200 μl of water. Tenfold serial dilutions of the cell suspensions were made, and 10 μl of each dilution was spotted onto synthetic complete plates (HC) and synthetic complete plates containing 0.1% 5-fluoroorotic acid (HC+5-FOA). The plates were then incubated for 3 days at 30°C unless otherwise indicated. In each case, at least three individual colonies of each strain were tested.

RT-PCR assays. Total RNA was isolated as described (54). Reverse transcription (RT)-PCRs were performed using Ready-To-Go RT-PCR beads (Pharmacia) according to the manufacturer's instructions. The primers and cycling parameters for the amplification of *MAT***a***1* RNA were identical to those described (57). Reaction products were resolved on 2% agarose gels and visualized with ethidium bromide.

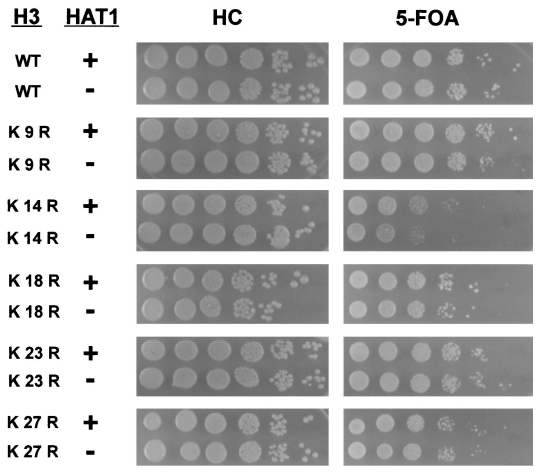

FIG. 1. Effects of *hat1*Δ and histone H3 single-lysine-to-arginine mutations on telomeric silencing. The indicated histone H3 alleles were introduced into UCC1111 (*HAT1*) and MPY1 (*hat1*Δ). Telomeric silencing was measured by spotting 10-fold serial dilutions of cells on synthetic complete plates (HC) and synthetic complete plates containing 5-FOA (HC+5-FOA) (Materials and Methods). Plates were photographed after 3 days of growth at 30°C. The relevant genotypes of the strains are indicated on the left. WT, wild type. Assays were performed on at least three separate colonies from each strain.

RESULTS

Hat1p and histone H3 are redundantly required for telomeric silencing. Yeast telomeric chromatin is capable of repressing the transcription of genes placed within several kilobases of the end of a chromosome (50). The integrity of this silent chromatin structure can be sensitively assayed using the *URA3* gene as a marker. Normal expression of the *URA3* gene from an internal chromosomal locus causes yeast cells to be sensitive to 5-FOA due to conversion of the 5-FOA into a toxin by the *URA3* gene product (7). When the *URA3* gene is placed near a telomere, expression of the gene is repressed in a high percentage of cells in the population (30 to 50%), allowing them to grow in the presence of 5-FOA. When telomeric silencing is disrupted, the *URA3* gene is expressed and the 5-FOA in the medium kills the cells (19).

The *HAT1* gene was deleted in a strain containing a telomeric *URA3* gene. As shown in Fig. 1, the absence of Hat1p had no effect on the level of telomeric silencing.

We next asked whether Hat1p had a role in telomeric silencing that was masked by functional redundancies. One candidate for such an effect is the histone H3 tail, which is required for telomeric silencing. While the role of the lysines in the H3 tail has not been examined in depth, mutating several of these residues has been shown to cause modest decreases in telomeric silencing that are synergistic with a mutation that alters lysines 5, 8, and 12 of the histone H4 tail (65).

We began our analysis by changing each of the five acetylatable lysines in the histone H3 NH_2-terminal tail to arginine. These changes preserve the basic charge of the residue but prevent it from being acetylated. Each allele was then introduced into wild-type and *hat1*Δ strains, and telomeric silencing was assayed as described above (Fig. 1). A number of observations can be made from this experiment. First, none of the lysine residues in the H3 tail was essential for telomeric silencing. Mutations at four of the histone H3 tail lysines (9, 18, 23, and 27) had little or no effect on telomeric silencing. Mutating the lysine at position 14 had the largest effect, causing a ≥10-fold reduction in silencing, as indicated by the decrease in the number of 5-FOA-resistant colonies. This is in contrast to the histone H4 tail, where mutation of lysine 16 completely abolishes telomeric silencing (4, 65). Also, while mutating individual lysine residues in the H3 tail did not uncover a significant *hat1*Δ phenotype, telomeric silencing was reduced further when *hat1*Δ mutations were combined with the H3 K14R allele (Fig. 1).

Next, we combined pairs of lysine-to-arginine mutations in the histone H3 tail with *hat1*Δ mutations. Five of the 10 histone H3 double-lysine-to-arginine mutations had little effect on telomeric silencing, even when they were combined with *hat1*Δ (Fig. 2). Two of the double histone H3 mutants showed decreased levels of silencing, independent of *HAT1*. However, three of the histone H3 double-lysine-to-arginine mutants had a much greater defect in telomeric silencing when the mutations were combined with a *hat1*Δ mutation (K9,14R, K14,23R, and K14,27R). In each case there was an approximately 100-fold effect on silencing. Interestingly, the *hat1*Δ phenotype required mutation of lysine 14 along with either lysine 9, 23, or 27. In each case when lysine 14 was unchanged, deletion of the *HAT1* gene had no effect. Therefore, pairs of lysine residues in the histone H3 tail that include lysine 14 appear to be functionally redundant with Hat1p for the formation of telomeric silent chromatin.

The *hat1*Δ-dependent decrease in growth on 5-FOA medium is specifically due to defects in telomeric silencing. *hat1*Δ/histone H3 mutants that lack a telomeric *URA3* gene grow normally on 5-FOA, indicating that these mutants are not hypersensitive to this drug. In addition, the *hat1*Δ/histone H3 mutants increased the expression of the *ADE2* gene when it was placed near the right arm of chromosome V, as deter-

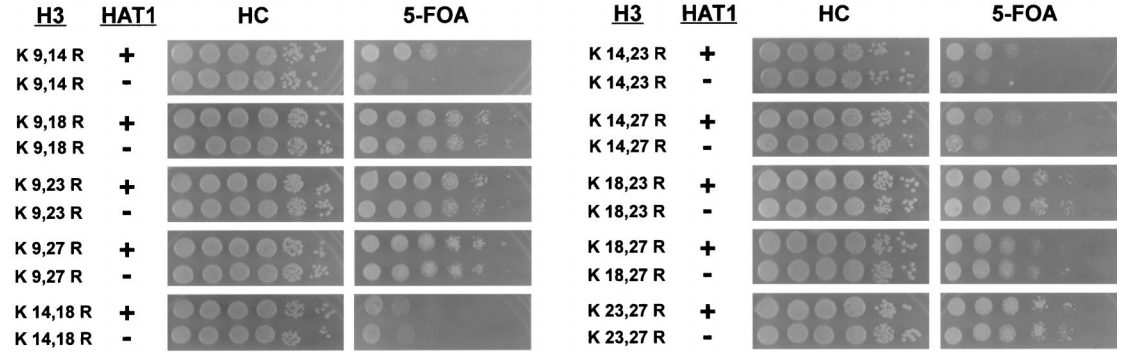

FIG. 2. Function of Hat1p in telomeric silencing is uncovered by particular histone H3 double-lysine-to-arginine mutations. The indicated histone H3 alleles were introduced into UCC1111 (*HAT1*) and MPY1 (*hat1*Δ). Telomeric silencing was assayed as described in the legend to Fig. 1. The genotypes are indicated on the left.

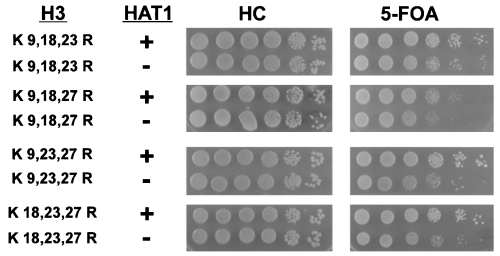

FIG. 3. *hat1*Δ causes a decrease in the size of 5-FOA-resistant colonies when combined with histone H3 triple-lysine-to-arginine mutations that retain lysine 14. The indicated histone H3 alleles were introduced into UCC1111 (*HAT1*) and MPY1 (*hat1*Δ). Telomeric silencing was assayed as in Fig. 1. The genotypes of the strains are indicated.

mined by changes in colony color, demonstrating that this phenotype was neither telomere nor gene specific (data not shown) (19).

Each histone H3 triple-lysine-to-arginine mutant combination was also assayed for telomeric silencing. In each case when lysine 14 was changed to arginine, there was a *hat1*Δ effect on telomeric silencing similar to that shown in Fig. 2 (data not shown). The major difference between the double and triple mutants was a general reduction in the overall level of silencing. A different phenotype was observed when deletions of *HAT1* were combined with H3 triple-lysine-to-arginine mutants that retain a lysine residue at position 14 (Fig. 3). While the number of 5-FOA-resistant colonies was unchanged, the size of the colonies was reduced dramatically in the *hat1*Δ background. This small-colony phenotype on HC+5-FOA plates was also observed for the H3 K14R mutant, independent of *hat1*Δ. The small-colony phenotype is not due to a general growth defect, as these strains grow normally in the absence of 5-FOA, but may be indicative of defects in the maintenance of the silent chromatin state (17, 42). Thus, a *HAT1*-dependent effect on telomeric silencing is observed when any three lysines are mutated to arginine in the histone H3 tail. However, the effect is modest if lysine 14 is not mutated.

Next we examined the effect on telomeric silencing when four of five lysines in the histone H3 tail were mutated to arginine. In two cases (K14,18,23,27R and K9,14,23,27R) telomeric silencing was virtually abolished (Fig. 4A). This precluded an evaluation in these strains of Hat1p in telomeric silencing. In the other three mutants, silencing was still evident and was reduced when *HAT1* was mutated. When lysine 14 remained (H3 K9,18,23,27R), mutating *HAT1* did not decrease the number of 5-FOA-resistant colonies but significantly reduced their size (the small *hat1*Δ colonies were more readily visible after 7 days of growth). When lysine 14 was changed to arginine (H3 K9,14,18,27R and H3 K9,14,18,23R), *hat1*Δ decreased both the number and size of the 5-FOA-resistant colonies.

An unexpected aspect of the results presented in Fig. 4A is that histone H3 alleles that contain only a single lysine residue at position 14, 23, or 27 retained a high level of telomeric silencing. We directly compared the level of telomeric silencing observed with wild-type histone H3 to the level of silencing seen with alleles that change either four or five lysines to arginine (Fig. 4B and C). Mutating all five lysines in the H3 tail reduced telomeric silencing to the detection limits of our assay. This level of silencing was comparable to that found with an allele that changes histone H4 lysine 16 to arginine, which has been well documented to cause a complete loss of telomeric silencing (4, 65). This demonstrated that, collectively, the acetylatable lysines in the histone H3 tail were essential for telomeric silencing. In addition, lysine 14 alone was sufficient to support a nearly wild-type level of telomeric silencing. When lysine 23 was the sole unchanged residue, the number of 5-FOA-resistant colonies was similar to that of wild-type histone H3, but the size of these colonies was greatly reduced. Lysine 27 was capable of supporting a moderate level of telo-

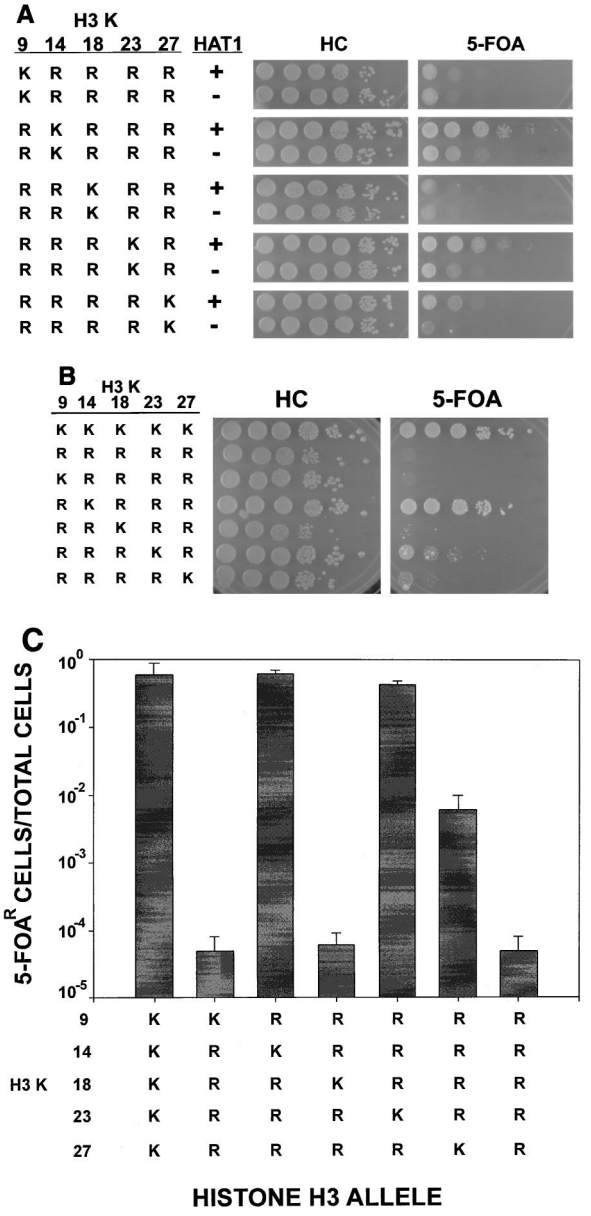

FIG. 4. Single histone H3 lysine residues can be sufficient to support telomeric silencing. (A) The indicated histone H3 alleles were introduced into UCC1111 (*HAT1*) and MPY1 (*hat1*Δ). Telomeric silencing was assayed as in Fig. 1. (B) Analysis of the level of telomeric silencing of histone H3 alleles containing four or five lysine-to-arginine mutations. The indicated histone H3 alleles were introduced into UCC1111. Telomeric silencing was assayed as in Fig. 1. (C) Telomeric silencing was quantitated from three to five repetitions of the assays shown in panels A and B by counting the number of colonies that grew on HC and HC+5-FOA plates. The mean has been plotted, with the error bars representing the standard deviation. Plates were incubated for 7 days to aid in the counting of small colonies.

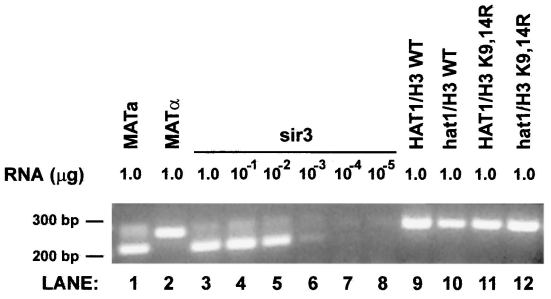

FIG. 5. HMR silencing is unaffected by *hat1*Δ/histone H3 mutations. RT-PCRs were performed on total RNA (without removal of genomic DNA) isolated from the MAT**a** strain BY4705a (lane 1) and MATα strains BY4705 (lane 2), UCC6580 (lanes 3 to 8), UCC1111 (lanes 9 and 11), and MPY1 (lanes 10 and 12). UCC1111 and MPY1 also contained the histone H3 alleles indicated. The amount of total RNA in each reaction is indicated. The migration of DNA molecular size standards is given on the left.

meric silencing, while lysines 9 and 18 were incapable of supporting telomeric silencing. Therefore, there is a hierarchy to the requirement for the acetylatable lysines in the histone H3 tail, where lysine 14 is the most effective, lysine 23 is a little less effective, lysine 27 is weakly effective, and lysines 9 and 18 are completely ineffective.

Hat1p does not influence HMR silencing. Telomeric heterochromatin involves many but not all of the factors involved in the transcriptional repression of the silent mating loci (4). The silent mating loci, *HML* and *HMR*, contain copies of yeast mating type-specific genes (**a** and α) that are expressed only when present at the *MAT* locus. The mechanism of transcriptional silencing at *HML* and *HMR* appears to be similar to the silencing of telomere-proximal genes (4, 51). However, *HML* and *HMR* silencing is stronger than telomeric silencing due to the presence of multiple *cis*-acting silencing elements and the participation of Sir1p in silencing at mating loci but not at telomeres (36).

We tested whether histone H3 and *hat1*Δ mutations derepress the *MAT***a***1* gene at the *HMR***a** locus. RT-PCR, using primers that flank a small intron, can be used to monitor expression of *MAT***a***1*. The RT-PCR product produced from mature *MAT***a***1* mRNA is approximately 50 bp smaller than the product derived from genomic DNA (57). *MAT***a***1* is normally expressed at the *MAT* locus in *MAT***a** cells and repressed in *MAT*α cells. This is visualized in the RT-PCR reactions shown in Fig. 5 (lanes 1 and 2), where the faster-migrating, mRNA-derived band is only generated from *MAT***a** RNA. As expected, *MAT***a***1* expression is completely derepressed in a *MAT*α *sir3*Δ cell (Fig. 5, lane 3). This RT-PCR has a 1,000-fold range of sensitivity, as determined by assaying 10-fold serial dilutions of the *MAT*α *sir3*Δ RNA (Fig. 5, lanes 3 to 8). In a *MAT*α strain, *hat1*Δ and H3 K9,14R mutations do not cause any apparent derepression of the *MAT***a***1* gene when it is silenced at *HMR***a**. Thus, as seen for the chromatin assembly factor Cac1p, the effects of Hat1p are more pronounced on telomeric silencing than on silent mating locus repression (17, 29, 42).

Hat1p affects telomeric silencing through histone H4 lysine 12. If the role of Hat1p in telomeric silencing is due to the acetylation of the histone H4 tail, then mutating lysine residues in the histone H4 tail might mimic the *hat1*Δ phenotypes. Like the *hat1*Δ mutation, individually mutating histone H4 lysines 5, 8, and 12 has no effect on telomeric silencing (data not shown). However, when the histone H4 mutations were combined with the histone H3 K9,14R allele (this allele produced the most pronounced *hat1*Δ phenotype with the fewest mutations in

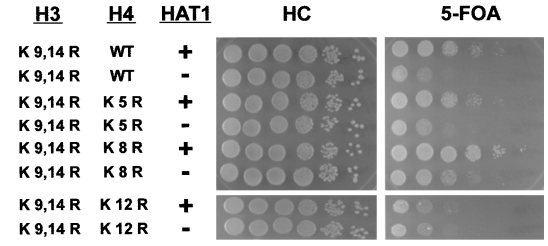

FIG. 6. Role of Hat1p in telomeric silencing is mediated through histone H4 lysine 12. The indicated histone H3-H4 alleles were introduced into UCC1111 (*HAT1*) and MPY1 (*hat1*Δ). Telomeric silencing was assayed as in Fig. 1.

histone H3), only the K12R mutant of histone H4 mimicked the *hat1*Δ effect on telomeric silencing (Fig. 6). Importantly, combining *hat1*Δ with the H3 K9,14R and H4 K12R alleles did not result in further decreases in telomeric silencing, suggesting that Hat1p is functioning through histone H4 lysine 12. In contrast, mutating histone H4 lysine 5 or 8 to arginine did not result in a decrease in telomeric silencing in the H3 K9,14R background. In fact, changing lysine 8 to arginine increased the level of telomeric silencing. This is consistent with the recent finding that deletion of *GCN5*, whose gene product acetylates histone H4 lysine 8, results in an increase in telomeric silencing (63). In addition, altering histone H4 lysines 5 and 8 did not affect the *hat1*Δ phenotype normally seen in combination with the H3 K9,14R allele. Taken together, these results suggest that Hat1p functions through histone H4 lysine 12.

Hat2p is involved in telomeric silencing. Hat2p was originally characterized biochemically as a positive regulatory subunit of the yeast histone H4-specific type B histone acetyltransferase complex (46). We took advantage of the *hat1*Δ telomeric silencing phenotype to test whether the regulation of Hat1p activity by Hat2p was also physiologically relevant. If Hat2p is required for the in vivo activity of Hat1p, deletion of the *HAT2* gene should cause a telomeric silencing defect similar to that of *hat1*Δ when combined with histone H3 mutations. In the presence of wild-type histone H3, *hat2*Δ caused little if any decrease in telomeric silencing (Fig. 7). However, combining the histone H3 K9,14R allele with *hat2*Δ resulted in a decrease in telomeric silencing similar to that seen with *hat1*Δ. Consistent with the idea that Hat1p and Hat2p function together, further decreases in the level of telomeric silencing were not observed in the *hat1*Δ *hat2*Δ double mutant. These results indicate that the Hat1p-Hat2p interactions identified in vitro are functionally relevant in vivo.

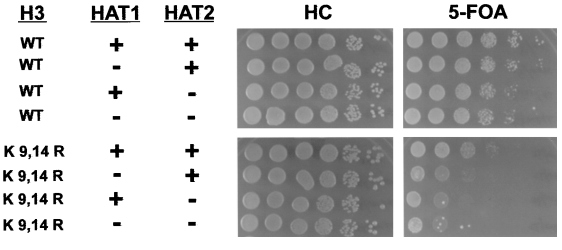

FIG. 7. Hat2p is involved in telomeric silencing. The indicated histone H3 alleles were introduced into UCC1111 (*HAT1*), MPY1 (*hat1*Δ), TKY101 (*hat2*Δ), and TKY104 (*hat1*Δ*hat2*Δ). The strains were then assayed for telomeric silencing as in Fig. 1.

DISCUSSION

We have identified a role for the Hat1p-Hat2p type B histone acetyltransferase complex in telomeric silencing. The function of this enzyme is redundant with the histone H3 NH_2-terminal tail, as the telomeric silencing defect of *hat1*Δ and *hat2*Δ strains is only apparent when particular lysine residues in histone H3 are also mutated.

In vivo characterization of Hat1p. Up to this point, our understanding of Hat1p has been based solely on in vitro biochemical analyses, due to a lack of obvious phenotypes in *hat1*Δ cells. A role for Hat1p in telomeric silencing now allows in vivo characterization of this enzyme. The fundamental question about the function of Hat1p, whether histone H4 is an in vivo substrate of this enzyme, can now be answered affirmatively. When the histone H3 gene is appropriately mutated, changing lysine 12 on histone H4 mimics the *hat1*Δ phenotype. This is completely consistent with earlier work in which Hat1p isolated from yeast cytoplasmic extracts specifically acetylates histone H4 only on lysine 12 (32, 46). Recombinant Hat1p isolated from *Escherichia coli* acetylates lysine 5 and lysine 12 of histone H4 as well as histone H2A. This relaxation in specificity may be a fortuitous trait of recombinant Hat1p or may reflect the fact that Hat1p specificity is regulated in yeast. Our present study on telomeric silencing is consistent with Hat1p acting only on lysine 12 of histone H4.

Furthermore, the current study indicates that Hat2p is critical to Hat1p activity in vivo; *hat1*Δ, *hat2*Δ, and combined *hat1*Δ *hat2*Δ mutations caused the same loss of telomeric silencing. These results are best explained by Hat2p's ability to stabilize the interaction of histone H4 with Hat1p, thus increasing the specific activity of Hat1p-mediated acetylation (46, 70).

Histone acetylation and heterochromatin. Histone H4 located in regions of silent chromatin in yeast or heterochromatin in *Drosophila* cells is acetylated in a distinct pattern. Lysines 5, 8, and 16 are hypoacetylated, and lysine 12 is acetylated at normal levels (10, 22, 67). It is unclear whether this acetylation pattern plays an active role in heterochromatin function, or whether this pattern is merely a consequence of the histones in heterochromatin being isolated from the histone acetyltransferases and deacetylases that normally act on euchromatin (22). Mutational analysis of the lysine residues of the H4 tail has produced contradictory results in terms of addressing the importance of this acetylation state for heterochromatic function. Mutating lysines 5, 8, and 12 to arginine, which is thought to mimic the constitutively unacetylated state, has little or no effect on transcriptional repression at *HML* and *HMR*, as measured by mating efficiency, or at telomeres (28, 45, 65). This suggests that these residues do not play a role in transcriptional silencing.

However, Braunstein et al. found that converting lysine 5 or 12 to glutamine, which has been suggested to mimic acetylated lysine, suppresses the mating defect seen when the other three histone H4 tail lysines are mutated to arginine (10). Enomoto and Berman found that converting lysines 5, 8, and 12 to arginine results in the formation of shmoo clusters when *MAT***a** cells are exposed to α-factor, indicative of subtle defects in *HML*α silencing (17). In addition, Hecht et al., using an in vitro assay to detect interactions between histone H4 tail peptides and Sir3p, found that mutating lysine 16 to glutamine, which eliminates silencing in vivo, has no effect on the histone H4-Sir3p interaction. It is only when lysine 12 (or 5 and 12) is also changed to glutamine that the histone H4-Sir3p interaction is disrupted, suggesting that lysine 5 or 12 provides part of the interaction of histone H4 with Sir3p (25). Our findings help to clarify these results and strongly support the idea that acetylation of lysine 12 plays an important role in the formation or maintenance of silent chromatin (heterochromatin). Until now, the role of this modification has been obscured by redundancy with the histone H3 tail. This "redundancy" may be explained by binding of silencing proteins, such as Sir3p, to both histone H3 and H4 tails.

The fact that Hat1p promoted a repressive chromatin structure runs counter to the action of other known histone acetyltransferases, which are uniformly involved in the activation of transcription. This may reflect a fundamental in vivo difference between type B and type A histone acetyltransferases, which were originally distinguished based solely on in vitro biochemical properties. One explanation for these results is that lysine 12 acetylation, which is carried out by Hat1p, is important for the interaction between histone H4 and a structural component of silent chromatin, such as Sir3p. The fact that the acetylation of a histone, which is generally thought to loosen the interaction between histones and DNA, can promote a repressive chromatin structure underscores the idea that the acetylation of histones can act as a signal that modulates protein-protein interactions (62).

In regions of silent chromatin, histone H3 is hypoacetylated (10). However, it is not known whether the hypoacetylation of the histone H3 lysine residues is uniform or whether there is a specific pattern of acetylation. Our demonstration that lysine-to-arginine mutations at specific sites can have dramatic effects on telomeric silencing suggests that the acetylation of histone H3 might play an important role in silent chromatin function. Alternatively, lysine per se at specific sites in histone H3 may be important for silencing. Whichever mechanism is at work, lysine 14 is a key player in silencing, and lysines 23 and 27 contribute to a lesser extent.

Definitive evidence for the involvement of histone H3 acetylation in silent chromatin will require an analysis such as we have carried out here for histone H4 and Hat1p-Hat2p. That is, it will require identification of a histone H3-specific acetyltransferase that is required for silent chromatin function. An obvious candidate is Gcn5p, which can acetylate lysine 14 of histone H3 in vitro (21, 33, 66). However, it was recently shown that *gcn5*Δ cells have increased levels of telomeric silencing (63). Other candidate acetyltransferases include Sas2p and Sas3p, which have been shown to be important for full telomeric silencing (49). Both proteins show limited homology to the GNAT superfamily of acetyltransferases (43).

Chromatin assembly and heterochromatin. As suggested above, Hat1p activity may be important in telomeric silencing because it acetylates lysine 12 of histone H4 and because acetylated lysine 12 is required for binding by silencing proteins (e.g., Sir3p). An alternative and mutually inclusive hypothesis is that acetylation of lysine 12 is required for interaction with a chromatin assembly factor, which ultimately leads to deposition of histone H4 for silent chromatin. One such potential chromatin assembly factor is CAF-1, which binds cytosolic histone H3-H4 tetramers and deposits them onto newly replicated DNA in vitro (30, 59). The histone H4 that is preferentially complexed with CAF-1 is acetylated on lysines 5, 8, and 12 (71). The fact that type B histone acetyltransferases, such as Hat1p, can acetylate free histone H4 on lysines 5 and 12 has led to the suggestion that Hat1p and CAF-1 operate in a common pathway, with Hat1p being at least partly responsible for acetylating the histone H4 that interacts with CAF-1 (2). Consistent with this model, mutants lacking the yeast CAF-1 subunits (*CAC1*, *CAC2*, and/or *MSI1*) display defects in telomeric silencing similar to those of the *hat1*Δ/histone H3 double mutants.

The pattern of acetylation of the histone H4 tail found in silent chromatin partially overlaps the acetylation pattern of newly synthesized histone H4. This has led to the idea that the modification present on histone H4 in silent chromatin is a result of the assembly of newly synthesized histones. However, if this is the case, why is heterochromatic histone H4 not acetylated at lysines 5 and 8? There are a number of possible explanations. CAF-1 may be capable of selectively interacting with and targeting populations of histones that have distinct acetylation states. In this way, CAF-1 may selectively assemble histone H4 acetylated at lysine 12 in regions of silent chromatin. Alternatively, histone deacetylases specific for lysines 5 and 8 may act in regions of silent chromatin to produce histones with the proper acetylation pattern. A third possibility is that the complete deacetylation of histone H4 following chromatin assembly that is seen in euchromatin also occurs in regions of silent chromatin. Hat1p may then reestablish the characteristic silent chromatin histone H4 acetylation pattern. This possibility is supported by the fact that Hat1p appears to be located in the nucleus as well as the cytoplasm (26, 46, 53, 70).

Thus, while we have demonstrated that the Hat1p-Hat2p histone acetyltransferase is important for telomeric silencing, the next challenge will be to determine when it plays its role in silent chromatin formation.

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