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Efficient Transcriptional Silencing in *Saccharomyces cerevisiae* Requires a Heterochromatin Histone Acetylation Pattern

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Heterochromatin in metazoans induces transcriptional silencing, as exemplified by position effect variegation in *Drosophila melanogaster* and X-chromosome inactivation in mammals. Heterochromatic DNA is packaged in nucleosomes that are distinct in their acetylation pattern from those present in euchromatin, although the role these differences play in the structure of heterochromatin or in the effects of heterochromatin on transcriptional activity is unclear. Here we report that, as observed in the facultative heterochromatin of the inactive X chromosome in female mammalian cells, histones H3 and H4 in chromatin spanning the transcriptionally silenced mating-type cassettes of the yeast *Saccharomyces cerevisiae* are hypoacetylated relative to histones H3 and H4 of transcriptionally active regions of the genome. By immunoprecipitation of chromatin fragments with antibodies specific for H4 acetylated at particular lysine residues, we found that only three of the four lysine residues in the amino-terminal domain of histone H4 spanning the silent cassettes are hypoacetylated. Lysine 12 shows significant acetylation levels. This is identical to the pattern of histone H4 acetylation observed in centric heterochromatin of *D. melanogaster*. These two observations provide additional evidence that the silent cassettes are encompassed in the yeast equivalent of metazoan heterochromatin. Further, mutational analysis of the amino-terminal domain of histone H4 in *S. cerevisiae* demonstrated that this observed pattern of histone H4 acetylation is required for transcriptional silencing. This result, in conjunction with prior mutational analyses of yeast histones H3 and H4, indicates that the particular pattern of nucleosome acetylation found in heterochromatin is required for its effects on transcription and is not simply a side effect of heterochromatin formation.

Heterochromatin, defined cytologically as regions of the genome that remain condensed throughout the cell cycle, can exert transcriptional repression (32). In *Drosophila melanogaster*, translocation of a euchromatic region of the genome to a site adjacent to heterochromatin often yields variable repression of the translocated genes. This repression results from heterochromatin spreading into the euchromatic domain, a process referred to as position effect variegation (17, 25, 75). In female mammalian cells, one of the two X chromosomes becomes heterochromatic early in development, which leads to heritable repression of most of its genes (11, 58, 60). The hallmark of both these cases of heterochromatin-induced regulation is that repression is position specific but gene nonspecific.

Certain loci in *Saccharomyces cerevisiae* are subject to long-term repression that is similar to repression associated with metazoan heterochromatin. Three separate loci on chromosome III—*MAT*, *HML*, and *HMR*—contain either **a** or α mating-type genes. At *MAT* these genes are expressed to specify the corresponding **a** or α cell type (27, 28). However, despite the fact that the promoters, coding sequences, and even flanking sequences resident at the *HM* loci are identical to those at *MAT*, the genes present at *HML* and *HMR* are fully repressed (38, 54). This repression, termed silencing, is position specific but gene nonspecific: insertion of different RNA polymerase II- and polymerase III-transcribed genes at the *HM* loci, or silent cassettes, results in their repression, and transposition of the mating-type genes out of the silent cassettes results in their activation (8, 28, 48, 64). Transcriptional repression of mating-type genes at the *HM* loci is critical to the maintenance of cellular identity since derepression of the silent cassettes would result in simultaneous expression of both **a** and α genes and a nonmating phenotype.

Elements essential for repression at *HML* and *HMR* have been defined genetically. Silencing depends on the concerted action of *cis*-acting sequences, known as the E and I silencers (1, 8, 18, 48), that flank *HML* and *HMR* and of several *trans*-acting factors, including the products of the *SIR* genes (21, 37, 61, 62). The E and I silencers are small (<250-bp) regions that confer *SIR*-dependent silencing on genes placed adjacent to them. Silencers consist of specific combinations of two or more sites for binding any of three DNA-binding proteins, and their silencing activity requires the binding of these proteins to their sites (4, 19, 40, 45, 46). Null mutations in the *SIR2*, *SIR3*, and *SIR4* genes lead to a complete loss of repression (30, 61, 65), although the functions of the products of the *SIR* genes are not known.

To explore the nature and function of the chromatin packaging the silent cassettes, we have examined the state of histone acetylation over this region. The amino-terminal domains of all four histones that compose the nucleosome core particle contain several lysine residues that are highly conserved and subject to reversible acetylation (16, 44, 72). Histone acetylation in vertebrate cells has been correlated with the potential for transcription; both active and inducible genes are packaged in acetylated nucleosomes, while genes that are subject to

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TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
Y851	*mat***a***1 HMLαHMRα leu2-3,112 ura3-52 ade8 trp1Δ901*	10
Y1422	*mat***a***1 HMLαHRMα leu2-3,112 ura3-52 ade8 trp1Δ901 sir2::TRP1*	10
Y82	*MATα his1*	Laboratory stocks
Y1794	*MAT***a** *ura3-52 leu2-3,112 lys2Δ201* Δ(*HHT1-HHF1*) Δ(*HHT2-HHF2*) [pMS329]	49
Y1990	*MAT***a** *ura3-52 leu2-3,112 lys2Δ201* Δ(*HHT1-HHF1*) Δ(*HHT2-HHF2*) [pMB31]	This study
Y1991	*MAT***a** *ura3-52 leu2-3,112 lys2Δ201* Δ(*HHT1-HHF1*) Δ(*HHT2-HHF2*) [pMB42]	This study
Y1992	*MAT***a** *ura3-52 leu2-3,112 lys2Δ201* Δ(*HHT1-HHF1*) Δ(*HHT2-HHF2*) [pMB44]	This study
Y2084	*MAT***a** *ura3-52 leu2-3,112 lys2Δ201* Δ(*HHT1-HHF1*) Δ(*HHT2-HHF2*) [pMB34]	This study
Y2085	*MAT***a** *ura3-52 leu2-3,112 lys2Δ201* Δ(*HHT1-HHF1*) Δ(*HHT2-HHF2*) [pMB32]	This study

long-term repression are packaged in relatively hypoacetylated nucleosomes (15, 22, 23, 55). Further, heterochromatic regions of metazoan genomes, which are generally transcriptionally silent, are generally hypoacetylated. In centric heterochromatin of *D. melanogaster*, three of the four acetylatable lysine residues of histone H4 (lysines 5, 8, and 16) are hypoacetylated relative to the lysine residues in active chromatin, although lysine 12 shows significant levels of acetylation (73). In addition, the lysine residues of histones H4 and H3 in the inactive X chromosome of mammals are generally hypoacetylated relative to the lysine residues of euchromatin (5, 6, 31). Whether acetylation levels or specific acetylated lysines influence transcription potential and, if so, how that effect is accomplished have not been resolved.

Previous studies have implicated histone deacetylation in transcriptional silencing in *S. cerevisiae*. Genetic analysis has suggested that the amino-terminal domain of histone H4—the region subject to reversible acetylation—is critical for silencing and that the corresponding domain of histone H3 contributes to the efficiency of the process (34, 35, 49, 56, 71). In addition, we have shown that the histone H4 packaging the silent cassettes is generally hypoacetylated relative to the histone H4 resident at active regions of the genome and that this hypoacetylation is a direct consequence of transcriptional silencing (10). As noted above, recent studies of metazoan heterochromatin have more precisely defined the nature of the acetylation patterns of histones associated with constitutive and facultative heterochromatin. Here we report that the acetylation patterns of histones H3 and H4 spanning the silent mating-type cassettes precisely match those found in metazoan heterochromatin. These results provide further evidence that silent cassettes are the yeast counterpart of metazoan heterochromatin. In addition, we show genetically that the pattern of histone H4 acetylation is important for efficient transcriptional silencing. This result, in conjunction with previous genetic analyses of histones H3 and H4 in *S. cerevisiae*, reveals that acetylation plays a substantive role in the silencing process.

MATERIALS AND METHODS

Plasmids and strains. Histone H4 mutants were created by site-directed mutagenesis with the Promega Altered Sites in vitro mutagenesis system on a 476-bp *Rsa*I fragment of *HHF1* flanked by *Bam*HI linkers. The mutagenized *hhf1* gene was sequenced in its entirety and cloned into *Bam*HI-cut pMS347, an *ARS1/CEN4* plasmid marked with *LEU2* and containing wild-type *HHT1* and a deletion of *HHF1*. Plasmid pMS347 is derived from plasmid pMS337 (52) by deletion of the *Bam*HI fragment spanning *HHF1*.

Plasmids pDM44 and pDM71 were used as sources of the *Hin*dIII-*Nde*I 246-bp α-specific and *Hin*dIII-*Eco*RI 296-bp **a**-specific probes, respectively, and have been previously described (48).

All yeast strains used in this study are listed in Table 1. Isogenic *SIR2* and *sir2* strains (Y851 and Y1422) were used for all chromatin immunoprecipitation experiments and have been previously described (10). All histone H4 mutations

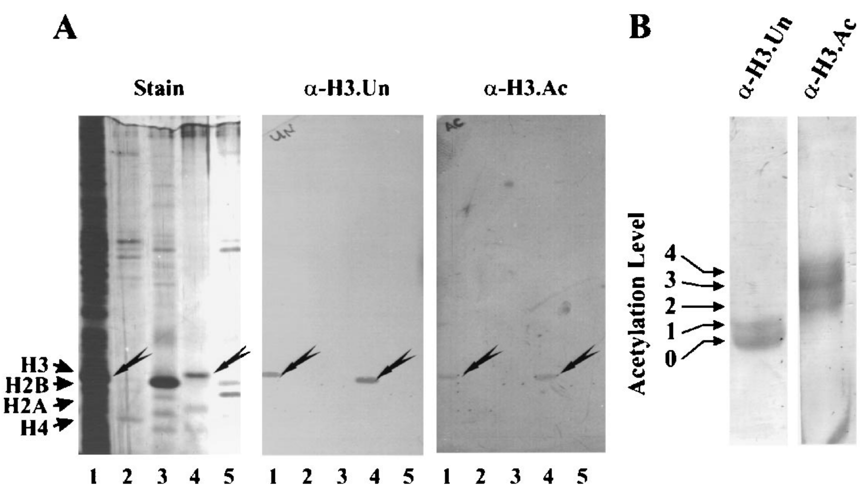

FIG. 1. Specificity of anti-histone H3 antibodies. (A) Total yeast chromatin proteins (ca. 15 to 20 μg) (lane 1) and 0.2 to 0.5 μg of high-pressure liquid chromatography-purified yeast histones H4 (lane 2), H2B (lane 3), H3 (lane 4), and H2A (lane 5) were fractionated by SDS-polyacrylamide gel electrophoresis and either silver stained (left panel) or transferred to polyvinylidene difluoride membranes and probed with anti-unacetylated-H3 antibody (α-H3 · Un) (1:250 dilution) or anti-acetylated-H3 antibody (α-H3 · Ac) (1:1,000 dilution). Bound antibody was visualized with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody. (B) Total yeast chromatin proteins were fractionated on a Triton-acetic acid-urea gel, transferred to polyvinylidene difluoride membranes, and probed with either anti-unacetylated-H3 antibody or anti-acetylated-H3 antibody. The positions of migration of histone H3 at the indicated levels of acetylation were determined from a parallel Coomassie-stained track.

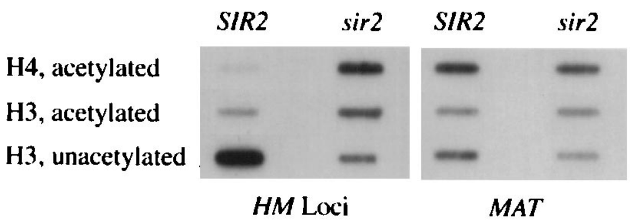

FIG. 2. Histone H3 is hypoacetylated in chromatin spanning the silent mating-type cassettes. Chromatin was prepared from strains Y851 (*SIR2*) and Y1422 (*sir2*) as described in Materials and Methods, and 1.0-ml samples were immunoprecipitated with either 10 μl of anti-acetylated-H4 antibody, 10 μl of anti-acetylated-H3 antibody, or 45 μl of anti-unacetylated-H3 antibody. DNA was isolated from the immunoprecipitates and applied to nitrocellulose with a slot blot manifold. Identical filters were then hybridized with an α-specific probe to identify *HML* and *HMR* sequences or with an **a**-specific probe to reveal *MAT* sequences. From control hybridizations whose results are not shown, both sets of sequences were equally represented in the unfractionated (total) chromatin preparation and none of the preimmune sera precipitated any detectable *HM* or *MAT* sequences.

were introduced into the host strain, Y1794 (equivalent to strain MX4-22A [49]), by plasmid shuffle.

Antibody preparation. The preparation of antibodies specific for particular acetylated lysine residues of histone H4 has been described elsewhere (74). Anti-acetylated-H4 antibodies have been previously described (43). Antibodies specific for acetylated and nonacetylated histone H3 were prepared with two synthetic peptides corresponding to the first 20 amino acids of H3 in either an unacetylated or diacetylated (lysines 9 and 14 were acetylated) form. These were conjugated to carrier protein (keyhole limpet hemocyanin) by using an artificial C-terminal cysteine residue and were injected into rabbits as previously described (43).

Histone preparation. Yeast histones were prepared by a modification of the procedure of Alonso and Nelson (2) described previously (10), and individual histones were purified by reverse-phase chromatography as described elsewhere (68). For visualization of different histone and chromatin fractions, samples were fractionated on sodium dodecyl sulfate (SDS)-polyacrylamide gels (12.5% polyacrylamide). For separation of different acetylated isoforms of histones, samples were fractionated on 17-cm Triton-acetic acid-urea gels (15% acrylamide, 0.1% bisacrylamide, 8 M urea, 5% acetic acid, 0.3% Triton X-100). After fractionation, proteins were visualized by silver staining or staining with Coomassie blue or by transfer to nitrocellulose and incubation with appropriate antisera. Blots were developed with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (1:1,000 dilution; Sigma).

Chromatin precipitation. For immunoprecipitation of solubilized chromatin (1 ml), prepared as described previously (10), the following were used: anti-acetylated histone H3 (10 to 12.5 μl); anti-unacetylated histone H3 (43 to 50 μl); antibodies to H4 acetylated at lysine 5 (20 to 25 μl; R41/5), lysine 8 (15 μl; R12/8), lysine 12 (25 to 43 μl; R20/12), and lysine 16 (38.5 to 50 μl; R14/K16); or anti-acetylated H4 (7.5 to 12.5 μl). The immune complexes were washed and collected, and the DNA present in the immunoprecipitates was isolated, extracted, and applied to a Nytran membrane by using a slot blot manifold as described previously (10). The presence of specific sequences in the immunoprecipitated material was determined by hybridization with ^{32}P-labeled genomic probes. The relative fraction of *HM* chromatin immunoprecipitated was determined by quantitation of probed blots with a Molecular Dynamics PhosphorImager and ImageQuant software. The fraction of the total *HM* chromatin immunoprecipitated was normalized to the fraction of *MAT* chromatin immunoprecipitated from each strain. Each value listed is the average from at least two immunofractionation experiments with the same antibody. Preimmune sera for the anti-acetylated-H3 antibody and the anti-unacetylated-H3 antibody did not immunoprecipitate any detectable *HM* or *MAT* DNA (data not shown).

Quantitative mating assays. Quantitative mating assays were performed essentially as previously described (48), using strain Y82 as the mating-type tester. Equal numbers of test and tester cells were mixed, filtered onto nitrocellulose, and incubated for 5 h at 30°C on the surface of a yeast extract-peptone-dextrose agar plate. Cells were then resuspended, and the numbers of diploids and haploid parents were determined by serial plating onto appropriate selective plates.

RESULTS

Histone H3 is hypoacetylated in chromatin spanning the silent cassettes. Since the inactive X chromosome in female mammalian cells exhibits reduced acetylation of histone H3 (5, 6), we explored whether reduced acetylation of H3 might be an additional characteristic shared between cytologically defined heterochromatin in metazoans and the *HM* loci in *S. cerevisiae*. To determine the acetylation status of histone H3 molecules located at the *HM* loci, we developed antisera that would specifically recognize either hypoacetylated or hyperacetylated histone H3. In one case, we raised polyclonal antibodies against a 20-amino-acid synthetic peptide corresponding to the amino-terminal domain of H3, in which lysine residues 9 and 14 were acetylated. As shown in Fig. 1A, this antibody recognizes only histone H3 in a yeast chromatin preparation. In addition, the antibody recognizes multiply acetylated isoforms of yeast histone H3 but not monoacetylated or unacetylated H3 isoforms (Fig. 1B). We also raised antibodies against the histone H3 peptide in which all lysine residues were unacetylated. This antibody recognizes histone H3 specifically and binds strongly to unmodified histone H3, weakly to monoacetylated histone H3, and not at all to multiply acetylated histone H3 (Fig. 1).

We used these antibodies to assess the extent of acetylation of histone H3 in nucleosomes that package specific portions of the yeast genome. Chromatin was isolated from various yeast strains. Immediately prior to harvesting, cells were briefly treated with formaldehyde to cross-link chromatin proteins to their contiguous DNA sequences. The purified chromatin was fragmented into small segments and then immunoprecipitated with the anti-histone H3 antibodies described above. The presence of specific DNA sequences within the immunoprecipitated chromatin could then be examined by reversing the cross-linking, extracting DNA from the precipitated nucleosomes, immobilizing the extracted DNA on nitrocellulose, and probing with sequences specific for the region in question.

To distinguish between silent and expressed mating-type loci in these experiments, we used a strain in which the *MAT* locus carried the **a** mating-type genes and both *HML* and *HMR* loci carried α mating-type genes. Thus, the fate of the silent locus sequences could be monitored by hybridizing with an α-specific probe, and the fate of the expressed *MAT* locus could be monitored with an **a**-specific probe. The relative proportion of the α-specific or **a**-specific sequences present in the precipitated chromatin to those in total chromatin thus reflected the extent to which the chromatin packaging these different mating-type loci was acetylated in vivo.

The results of our chromatin fractionation analysis are

TABLE 2. Acetylation state of histone H3 in nucleosomes packaging the silent cassettes[a]

Chromatin fraction	Relative fraction of *HM* chromatin in strain:	
	Y851 (wild type)	Y1422 (*sir2*)
Acetylated histone H3	0.58	0.99
Deacetylated histone H3	2.65	0.86
Acetylated histone H4	0.13	0.98

[a] Chromatin was isolated from strains Y851 and Y1422 and fractionated by immunoprecipitation with each of three antibodies: anti-acetylated H3, anti-unacetylated H3, and anti-acetylated histone H4. The relative fraction of *HM* chromatin present in each of the resultant chromatin fractions is presented. The relative fraction of *HM* DNA immunoprecipitated was determined by quantitation of probed blots like that shown in Fig. 2. The values shown were calculated by first determining the fraction of *HM* DNA precipitated by antibody relative to the amount present in an equivalent sample of total, unfractionated chromatin. This value was then normalized to the relative fraction of *MAT* DNA precipitated by the same antibody. Thus, the amount of *MAT* DNA precipitated by the antibody is defined as 1.0. The values are the averages from two separate immunofractionation experiments.

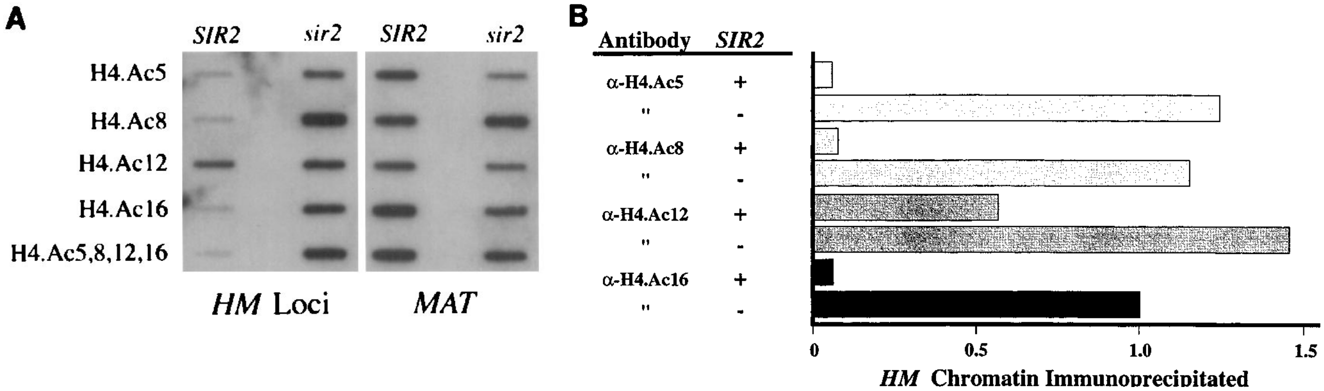

FIG. 3. Histone H4 in chromatin spanning the silent mating-type cassettes exhibits a specific pattern of acetylation. (A) Chromatin was prepared as described in Materials and Methods and then fractionated by immunoprecipitation with different antisera, of which some were specific for histone H4 molecules in which the indicated lysine residue was acetylated (e.g., H4.Ac5 indicates a histone H4 molecule in which lysine 5 is acetylated) and others generally recognized multiply acetylated histone H4. The presence of the *HM* loci and *MAT* locus in the chromatin fraction precipitated by each antiserum was determined by extracting DNA from the fraction, applying it to a Nytran filter in a slot blot array, and hybridizing it with a DNA probe specific for *MAT* or the *HM* loci. (B) The absolute fraction of *HM* chromatin immunoprecipitated was determined by quantitation of blots like those shown in panel A and normalized to the fraction of *MAT* chromatin immunoprecipitated in each strain. The values are the averages of four separate experiments.

shown in Fig. 2 and summarized in Table 2. The fraction of yeast chromatin immunoprecipitable with the anti-acetylated-H3 antibody contained less *HM* locus DNA than *MAT* locus DNA. Conversely, the fraction of chromatin immunoprecipitable with the anti-unacetylated-H3 antibody contained more *HM* locus DNA than *MAT* locus DNA. The results of these two experiments are consistent and indicate that histone H3 in nucleosomes spanning the *HM* loci is less acetylated than that in nucleosomes spanning the *MAT* locus. The selective precipitation of *MAT* versus *HM* loci with anti-acetylated-H4 antibodies is more dramatic than with anti-acetylated-H3 antibodies. This may reflect either a greater differential in the relative extent of hypoacetylation of the two histones at the silent cassettes or a lower discrimination potential of the anti-acetylated-H3 antibodies for hypo- versus hyperacetylated chromatin. The fact that the anti-acetylated-H3 antibodies were obtained against a peptide that was only diacetylated, rather than tetraacetylated, as was the case for the anti-acetylated-H4 antibody, lends credence to the second possibility. Nonetheless, we conclude that nucleosomes spanning the silent loci are substantially hypoacetylated on histone H3 as well as histone H4.

These results also provide evidence that the amino-terminal tails of histones at the *HM* loci are accessible to antibodies, since anti-unacetylated-H3 antibody precipitates a larger fraction of *HM* locus DNA than of *MAT* DNA. Thus, the reduced amount of *HM* DNA in precipitates obtained with anti-acetylated-H3 antibodies reflects reduced acetylation of the nucleosomes rather than any reduced accessibility. This observation fortifies our conclusion from previous studies on histone H4 that the reduced immunoprecipitability of *HM* locus nucleosomes with anti-acetylated-H4 antibodies reflects reduced histone H4 acetylation across the silent cassettes (10). Accordingly, like the heterochromatic, inactive X chromosome in mammalian cells, the silent cassettes are packaged in nucleosomes that are hypoacetylated on both histones H3 and H4.

Hypoacetylation of histone H3 at the *HM* loci is dependent on the transcriptional silencing mechanism. As evident in Fig. 2 and Table 2, the level of *HM* locus DNA precipitable from chromatin isolated from a *sir2* strain by using either anti-acetylated-H3 or anti-unacetylated-H3 antibodies is equivalent to that from *MAT*. Thus, hypoacetylation of H3 at the *HM* loci requires an intact transcriptional silencing apparatus. Although we have not tested directly for H3 acetylation, our previous results for H4 acetylation suggest that differential acetylation in Sir^+ and Sir^- strains is not an indirect consequence of differences in transcription; rather, hypoacetylation is likely a direct effect of silencing.

Histone H4 at the silent loci exhibits a specific pattern of acetylation. Histone H4 in centric heterochromatin from *D. melanogaster* is generally hypoacetylated but not equally so on all lysine residues in the amino-terminal tail. Rather, lysine residues 5, 8, and 16 are hypoacetylated, while lysine 12 shows nearly normal levels of acetylation (73). To determine whether a similar pattern of acetylation of histone H4 exists in chromatin spanning the silent cassettes, we used four different antisera, each specific for one of the acetylated lysines of histone H4. The specificity of each antibody preparation for the acetylated lysine residue against which it was raised has been documented previously (73, 74). These antibodies specifically recognize yeast histone H4, as detected by immunoblotting of yeast nuclear proteins (14). In addition, the specificities of these antibodies in immunoprecipitation have been demonstrated (55).

We used these residue-specific antibodies in an experiment

TABLE 3. Requirement for specific neutral lysines in histone H4 for efficient transcriptional silencing[a]

Plasmid	Amino acid at position:				Mating efficiency	*n*
	5	8	12	16		
pMB32	K	K	K	K	1.0	NA[b]
R5R8R12R16[c]	R	R	R	R	0.0009	
pMB42	Q	R	R	R	0.47	5
pMB34	R	Q	R	R	0.06	5
pMB31	R	R	Q	R	0.57	10
pMB44	R	R	R	Q	0.0001	4

[a] Quantitative mating assays were performed on strains carrying the indicated histone H4 mutations, and the mating efficiency was normalized to that obtained with the same strain carrying plasmid pMB32, determined at the same time. The reported mating efficiency is the average value from a number (*n*) of independent transformants tested.

[b] NA, not applicable.

[c] Plasmid R5R8R12R16 was described previously (56), and the mating efficiency is the published value.

similar to that described in the previous section to immunoprecipitate that portion of yeast chromatin whose nucleosomes were acetylated at a specific histone H4 lysine residue. The fraction of total *HM* locus chromatin that was immunoprecipitated by any one of these antisera revealed the acetylation status of the specific lysine residue recognized by that antibody. We found that lysines 5, 8, and 16 are substantially underacetylated in nucleosomes spanning the silent mating cassettes relative to those spanning the *MAT* locus (Fig. 3). In contrast, lysine 12 shows significant levels of acetylation. Hypoacetylation of histone H4 lysine residues of nucleosomes spanning the *HM* loci requires an intact silencing apparatus: mutation of the *SIR2* gene eliminates histone H4 hypoacetylation in the nucleosomes packaging these loci (Fig. 3). This pattern of histone H4 acetylation is consistent with the prediction, based on genetic analysis, that lysine 16 must be deacetylated in order to support transcriptional repression at the silent cassettes (34, 56). In addition, the fact that yeast silenced chromatin and *Drosophila* centric heterochromatin exhibit identical patterns of histone H4 acetylation provides compelling evidence that silenced domains are the yeast counterpart of metazoan heterochromatin.

The pattern of histone H4 acetylation is required for efficient transcriptional silencing. This similarity between the patterns of acetylation at the silent cassettes in yeast chromatin and those in heterochromatin in metazoans prompted us to determine the relevance of the specific acetylation pattern to the silencing mechanism. We constructed a variety of mutants in which the acetylatable lysines in histone H4 were specifically altered and then assessed the effect of these alterations on silencing. This was accomplished by determining the efficiency of mating of the various mutant strains, since derepression of the silent cassettes yields a nonmating **a**/α phenotype whereas repression of the *HM* loci retains the mating capacity of a cell. As shown in Table 3, Park and Szostak demonstrated that yeast strains whose sole H4 gene carries multiple mutations converting all four acetylatable lysines to arginines (lysine-to-arginine change at positions 5, 8, 12, and 16 (K5R, K8R K12R K16R), thus mimicking histone H4 in the fully deacetylated state, show a significant loss of transcriptional silencing of the *HM* loci (56). This suggests that a fully deacetylated histone H4 does not support silencing and prompted us to examine mutant histone H4 genes in which one of the lysines was mutated to glutamine, mimicking an acetylated residue. As shown in Table 3, we found that a strain that carries a histone H4 gene mutated to mimic the acetylation state observed at the silent cassettes (K5R K8R K12Q K16R), and that differs from the previous mutant by a single glutamine-for-arginine substitution at lysine 12, exhibits almost normal levels of transcriptional silencing. This difference in the ability of the two mutant histone genes to support transcriptional silencing is not simply a charge effect. Although a strain carrying the histone H4 mutation K5Q K8R K12R K16R also exhibits normal transcriptional silencing, the same strain carrying the histone H4 mutation K5R K8Q K12R K16R shows a 10-fold decrease in transcriptional repression of the *HM* loci, and the histone H4 mutation K5R K8R K12R K16Q, as previously reported (24), has an even more dramatic silencing defect. Since strains carrying any of these four mutant histone H4 genes grow equally well, the silencing differences of the four mutant constructs cannot be attributed to indirect effects of the mutants on the general viability of the cells. These results indicate that certain patterns of charged and neutral lysines in the amino terminus of histone H4 are required for efficient silencing: lysine 16 must be deacetylated, as noted previously (34, 56), and at least lysine 5 or lysine 12 must be acetylated. In vivo, yeast cells use lysine 12 for this requisite acetylation. These results indicate not only that a particular pattern of histone acetylation provides a biochemical marker for heterochromatin but also that this pattern is important for the effects of heterochromatin on transcription in *S. cerevisiae* and, by implication, in metazoans.

DISCUSSION

Silenced domains in *S. cerevisiae* are heterochromatin. A variety of previous observations have demonstrated that repression of the silent cassettes is accomplished through some form of altered chromatin structure across the region. First, the silent cassettes are relatively inaccessible to DNA-modifying agents, both in vivo and in chromatin preparations in vitro (47, 53, 66, 69, 70). Second, nucleosomes spanning the silent cassettes and other silenced regions fractionate on mercury affinity columns in a pattern similar to that of inactive chromatin (12, 13). Third, several genetic observations suggest a role for histones and histone modification in transcriptional silencing (33–35, 49, 56, 71). Fourth, yeast telomeres, whose metazoan counterparts are heterochromatic, promote position effect repression using the same cellular machinery as that responsible for transcriptional silencing of the mating-type cassettes (3, 20). Finally, silencing acts through a region-specific but sequence-nonspecific mechanism (8, 38, 48, 54, 64), which is similar to that observed in metazoan heterochromatin. These observations are all consistent with the hypothesis that the silent loci are rare instances of heterochromatic domains in *S. cerevisiae*.

The studies presented here confirm this hypothesis by demonstrating that the signature patterns of acetylation for metazoan heterochromatin are present in chromatin spanning the yeast silent cassettes. Certain patterns of histone acetylation are specifically correlated with cytologically defined heterochromatin in metazoans. For instance, the inactive X chromosome in mammals is packaged in nucleosomes in which both histones H3 and H4 are hypoacetylated (5, 6, 31). We found that the same is true of chromatin over the yeast silent cassettes. In addition, the particular pattern of histone H4 acetylation in *Drosophila* centric heterochromatin (73) is precisely recapitulated in the chromatin of yeast silent DNA. Although we have no evidence that H4 lysine 12 remains acetylated in the facultative or constitutive heterochromatin of mammals (5, 31), recent findings show that the level of acetylation of H4 lysine 16 in such heterochromatin is significantly higher than those of the other three lysines (36). Perhaps the continued acetylation of lysine 16 plays a role in mammalian heterochromatin similar to that of lysine 12 in *D. melanogaster* and *S. cerevisiae*. Reinforcing the validity of hypoacetylation as a marker for heterochromatin in *S. cerevisiae* is the fact that hypoacetylation in *S. cerevisiae* is strictly associated with loci subject to position effect repression. Neither active genes, uninduced repressible genes, nor even long-term-repressed genes—such as cell-type-specific genes in the nonexpressing cell type—are packaged in hypoacetylated nucleosomes in *S. cerevisiae* (9, 10). Thus, acetylation patterns provide a surrogate marker that allows us to identify heterochromatic regions in organisms not readily amenable to cytological analysis.

Formation of the nucleosome acetylation pattern. Several mechanisms could explain how the specific pattern of histone H4 acetylation is achieved. One possibility is that the pattern is attained by specific acetyltransferases and deacetylases acting on the nucleosomes present at the silent cassettes. For instance, an acetyltransferase specific for lysine 16 and restricted to the X chromosome has been invoked to account for the enhanced lysine 16 acetylation associated with the hyperactive

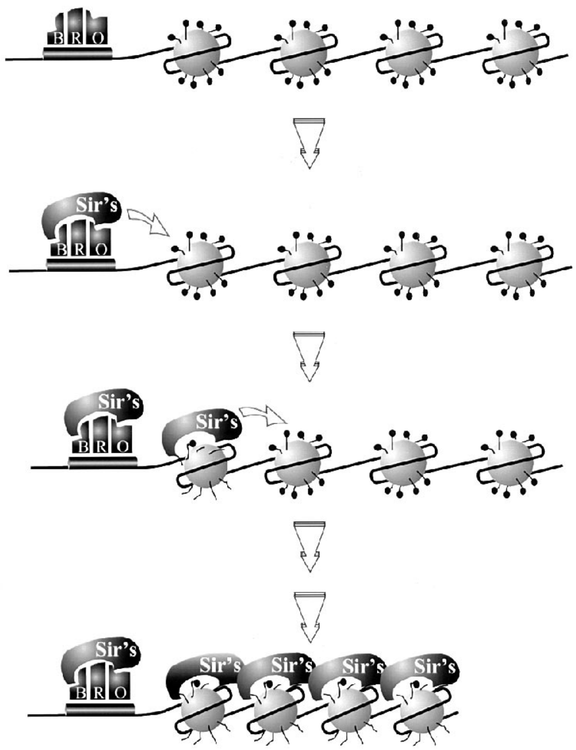

FIG. 4. Proposed role for acetylation in silencing. A model for silencer-induced heterochromatin formation is presented. Proteins that bind the silencers—Rap1p (R), Abf1p (B), and ORC (O)—recruit additional proteins—Sir2p, Sir3p, and Sir4p (Sir's)—that are themselves responsible for the formation of the heterochromatic structure in the region adjacent to the silencer. These Sir proteins induce a specific pattern of acetylation in the adjacent nucleosome, which increases the affinity of the nucleosome for the Sir proteins. The Sir proteins then bind to the newly modified nucleosome and induce modification of the adjacent nucleosome. In this manner, the silencing domain can be propagated outward from the nucleation site. The Sir proteins remain associated with the nucleosomes and participate directly, either as structural components or in a catalytic capacity, in the heterochromatin structure.

X chromosome in male *D. melanogaster* (7, 41, 73). Thus, in this model the final pattern of acetylation would emerge from a dynamic competition among nucleosome acetyltransferases and deacetylases, at least one of which was restricted to the silenced regions of the genome.

An alternative model suggests that the pattern of nucleosome acetylation at the silent cassettes is a remnant of the pattern of histone acetylation that precedes deposition. Deposition-related acetylation of histone H4 is conserved among many different organisms and involves cytoplasmic acetylation of histone H4 on lysines 5 and 12 (68). A variation on this theme would posit the existence of a pool of histones destined for deposition that were acetylated only on lysine 12. The identification of cytosolic histone acetyltransferase activities in *S. cerevisiae* and *D. melanogaster* that acetylate histone H4 only on lysine 12 lends some credence to this model (39, 67). In this case, persistence of the pattern of histone H4 acetylation at the silent cassette would simply involve recruitment of these monoacetylated histones to the newly replicated loci, followed by complete restriction of access of acetyltransferases and deacetylases to the deposited nucleosomes. The general inaccessibility of chromatin spanning the silent cassettes to various modifying enzymes (47, 53, 66, 69, 70) is consistent with this model.

It has been shown in this and in a previous study (10) that Sir2p, as well as Sir3p and Sir4p, is required for the persistence of the hypoacetylated state of the silent cassette and that overexpression of Sir2p, but not of the other silencer proteins, yields a general reduction in the levels of histone acetylation in yeast chromatin. As evident in the previous study, Sir2p overexpression effects hypoacetylation of all histones (10). Thus, Sir2p is the component of the silencing apparatus that is primarily responsible for hypoacetylation of the silent domains. This effect of Sir2p could be explained by proposing that it is targeted to the silent cassettes and acts as a histone deacetylase or an inhibitor of a histone acetyltransferase. An alternative, and perhaps more likely, explanation is that Sir2p restricts access, directly or indirectly, of the nucleosome acetyltransferases to chromatin with which Sir2p is associated. We have shown that Sir2p interacts with Sir3p (29, 63), which, since Sir3p is targeted to silencers through interaction with silencer-binding proteins (51), provides a mechanism to restrict Sir2p, and the specific pattern of histone acetylation, to the silenced regions of the yeast genome.

A role for acetylation in transcriptional silencing. The pattern of histone acetylation observed at the silent cassettes not only marks the region as heterochromatin but also has functional consequences for silencing. Previous analyses demonstrated that a loss of the positive charge at lysine 16 by mutation and, by implication, by acetylation fully abolished transcriptional silencing (34, 56). However, in the context of a normal lysine 16 residue, mutation of none of the other lysines had an effect on silencing (34, 49, 56). In this report we have shown that in the context of a fully hypoacetylated histone H4, either lysine 5 or lysine 12 must be acetylated for efficient silencing. This is not merely an issue of charge, since neutralizing lysine 8 does not yield efficient silencing. In related studies, Thompson et al. (71) have shown that histone H3 mutants designed to resemble unacetylated histones support transcriptional silencing in yeast strains much better than histone H3 mutants designed to resemble acetylated H3. Accordingly, we conclude that the pattern of acetylation observed in heterochromatin in *S. cerevisiae* and, by extension, in metazoans promotes efficient transcriptional repression of the underlying genes.

We note that the requirement for an acetylated lysine 12 in silencing is not absolute. That is, while histone mutants RRRK (with the mutation K5R K8R K12R K16), KKKR, and RRQR are not defective for silencing, both RRRR and RRRQ are defective (34, 56; also this report). A likely interpretation of this result is that when the first three lysine residues are fixed in the nonacetylated state, lysine 16 must be free to cycle between acetylated and unacetylated forms in order for the histone to function in silencing (cf. reference 50), whereas when lysine 5 or lysine 12 is fixed in the acetylated state, the constraints on lysine 16 are reduced and this residue does not have to cycle between forms. In other words, these mutations exhibit a synthetic phenotype. When one site (K5 or K12) is mutated, the second site (K16) is absolutely constrained; when the second site is mutated, the first site is absolutely constrained. As with synthetic lethality between two genes, this suggests that both sites play a role in the affected biological process, which in this case is silencing.

The fact that a particular pattern of acetylation is necessary for the transcriptional effects suggests the requirement for specific protein interactions between the nucleosomes and components required for transcriptional silencing. Sir3p and Sir4p, proteins essential for transcriptional silencing in *S. cerevisiae*, are potential candidates for such components, since they have recently been shown to bind to the amino-terminal domains of unmodified histones H3 and H4 (24). An intriguing possibility is that the acetylation state of the lysines in the amino termini affects the affinity of these proteins for binding to nucleosomes. While the effects of acetylation of histone H3 or of the other lysine residues of histone H4 have not been examined, Hecht et al. (24) have shown that the charge state of lysine 16 affects the binding of Sir3p and Sir4p to the amino-terminal domain of histone H4.

The prospect that acetylation might affect the affinity of Sir3p and Sir4p for nucleosomes in chromatin prompts a model that could help account for both heterochromatin spreading (26, 58, 59) and inheritance of the silenced state (57, 60) (Fig. 4). That is, Sir3p and Sir4p, recruited to the nucleation site through interaction with one or more of the silencer-binding proteins, in turn recruit Sir2p, which imparts the unique pattern of histone acetylation to the adjacent nucleosomes. This pattern of acetylation would facilitate the binding of Sir3p and Sir4p to the newly modified nucleosome, Sir3p and Sir4p would again recruit Sir2p to modify the adjacent nucleosome, and the process would continue. In this manner, the silencing domain can be propagated outward from the nucleation site. The Sir proteins likely remain associated with the nucleosomes and participate directly, either as structural components or in a catalytic capacity, in the heterochromatin structure. This mechanism could also contribute to inheritance of the silenced state. Newly deposited histones situated adjacent to silenced nucleosomes that had been randomly distributed to daughter chromatids following replication would be modified by Sir2p associated with the old nucleosomes. The new nucleosome would thereby acquire enhanced affinity for the silencing complex, and silencing would be reestablished in the new generation. Clearly, further analysis of the dependence of particular protein-histone interactions on histone acetylation should provide useful data to address the role of acetylation patterns in transcriptional silencing and help clarify the underlying mechanism of transcriptional repression by heterochromatin in eukaryotes.

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