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Deposition-related sites K5/K12 in histone H4 are not required for nucleosome deposition in yeast

(nucleosome assembly/acetylation/*Saccharomyces cerevisiae*)

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ABSTRACT **Histone H4 can be acetylated at N-terminal lysines K5, K8, K12, and K16, but newly synthesized H4 is diacetylated at K5/K12 in diverse organisms. This pattern is widely thought to be important for histone deposition onto replicating DNA. To investigate the importance of K5/K12 we have mutagenized these lysines in yeast and assayed for nucleosome assembly. Assaying was done in the absence of the histone H3 N terminus, which has functions redundant with those of H4 in histone deposition. Nucleosome assembly was assayed by three methods. Because nucleosome depletion may be lethal, we examined cell viability. We also analyzed nucleosome assembly *in vivo* and *in vitro* by examining plasmid superhelicity density in whole cells and supercoiling in yeast cell extracts. All three approaches demonstrate that mutagenizing K5 and K12 together does not prevent cell growth and histone deposition *in vivo* or *in vitro*. Therefore, K5/K12 cannot be required for nucleosome assembly in yeast. It is only when the first three sites of acetylation—K5, K8, and K12—are mutagenized simultaneously that lethality occurs and assembly is most strongly decreased both *in vivo* and *in vitro*. These data argue for the redundancy of sites K5, K8, and K12 in the deposition of yeast histone H4.**

Nucleosome assembly involves the deposition of a tetramer of histones H3 and H4 onto DNA, followed by the association of two histone H2A/H2B dimers. In this process, acetylation of histone H4 is likely to play a key role. Newly synthesized histone H4 was shown by pulse-labeling to be diacetylated at lysine residues 5 and 12 (K5/K12), a conserved feature in *Tetrahymena*, flies, and humans (1–3). This distinct nonrandom pattern of acetylation among the four acetylatable lysines (K5, K8, K12, and K16) has led to the suggestion that K5/K12 diacetylation serves a unique role in targeting newly synthesized histone H4 for assembly (4). Another argument for the importance of H4 acetylation in nucleosome assembly derives from the study of the human multiprotein complex (CAF-1, for chromatin assembly factor) that enables H3 and H4 assembly onto replicating DNA in a simian virus 40-based cell-free system (5–7). CAF-1 deposits newly synthesized histones but not those extracted from bulk chromatin onto DNA (7), a result which is consistent with the finding that CAF-1 associates preferentially with histone H4 acetylated in a specific manner (8). However, H4 associated with CAF-1 is not uniquely diacetylated at K5 and K12 but is heterogeneously acetylated at K5, K8, and K12. Moreover, some 33% of H4 in the complex is not acetylated. In addition, much of the H3 in the complex is monoacetylated, whereas some 60% of H3 is unacetylated (9).

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The yeast (*Saccharomyces cerevisiae*) histone H4 N-terminal sequence and the location of its acetylated lysines are extremely conserved in evolution. While it is not known whether newly synthesized yeast H4 is diacetylated or whether acetylated H4 is associated with a yeast chromatin assembly factor, we set out to ask whether K5/K12 is required for nucleosome assembly in yeast. Because the H3 N terminus can substitute for the H4 N terminus to allow cell viability and nucleosome assembly (10–12), we analyzed H4 N-terminal mutations in a genetic background in which the H3 N terminus was also deleted. The experiments presented here show that the presence of H4 K5/K12 (and by inference, K5/K12 diacetylation) is not required for cell viability or nucleosome assembly *in vivo* or *in vitro*. Instead, any of the first three sites K5 or K8 or K12 can support yeast histone H4 assembly in a redundant manner. Our data are consistent with the association of K5-, K8-, or K12-acetylated H4 with the human CAF1 complex. This relationship argues that acetylation of any of the sites K5, K8, or K12 provides a signal for the recognition of newly synthesized H4 by the chromatin assembly machinery.

MATERIALS AND METHODS

Mutations. For plasmid constructions and PCRs standard techniques were followed (13). The *Eco*RI/*Bam*HI fragments containing H4 mutations constructed previously (14–16), Δ4–14; Δ4–19; K5,8,12G; K8,12,16G; and K5,8,12,16G, were subcloned into pRM430 (17) to replace the wild-type H4 gene, resulting in various H3/H4 double-mutant plasmids. The *Eco*RI/*Sal*I fragments containing both H3 and H4 genes from these plasmids and pRM430 were then subcloned into pRS317 (18), a *CEN ARS LYS2* plasmid, yielding the pMX plasmids (see Table 1). The mutations K5,12G; K5,12R; K5,8R; K5,12,16G; and K5,8,16G were constructed by the megaprimer PCR method (19) using pLD101 (14) (*hhf2-K5R*) as template. The *Eco*RI/*Bam*HI fragments containing these H4 mutations were subcloned into pRM430 to replace the wild-type H4 gene. All constructed mutations were confirmed by dideoxynucleotide sequencing (United States Biochemical/Amersham).

Yeast Strains. The strains used in this study are listed in Table 1. All strains were derived from RMY430, which was described previously (17), As indicated in Fig. 1, in addition to plasmid B, which carries various H3/H4 mutations, these strains also contain pRM102, in which wild type H3 and H4 genes are under control of the divergent *GAL1–10* promoter (17), and were maintained in galactose-containing media. These constructs allowed us to analyze the mutant histone phenotypes by shifting cultures from galactose to glucose. All strains were grown at 30°C. Synthetic media lacking trypto-

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Abbreviation: CAF-1, chromatin assembly factor 1.
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Table 1. Strains used in this study

Strain	Genotype
MAY200	*MATa ade2-101 his3-Δ201 lys2-801 trp1-Δ901 ura3-52* (*hht1 hhf1*)::*LEU2* (*hht2 hhf2*)::*HIS3* plus pRM200 (*CEN4 ARS1 TRP1 HHT2-HHF2*) and pRM102 (*CEN4 ARS1 URA3 pGAL10-HHT2 pGAL1-HHF2*)
RMY430	*MATa ade2-101 his3-Δ201 lys2-801 trp1-Δ901 ura3-52* (*hht1 hhf1*)::*LEU2* (*hht2 hhf2*)::*HIS3* plus pRM430 (*CEN4 ARS1 TRP1 hht2-Δ430 HHF2*)
MAY430	Same as RMY430, except pMX430 (*hht2-Δ430 HHF2* cloned into pRS317) in place of pRM430, plus pRM102 (*CEN4 ARS1 URA3 pGAL10-HHT2 pGAL1-HHF2*)
MAY817	Same as MAY430, except pMX817 (*hht2-Δ430, hhf2-Δ414*)
MAY818	Same as MAY430, except pMX818 (*hht2-Δ430, hhf2-Δ419*)
MAY502	Same as MAY430, except pMX502 (*hht2-Δ430, hhf2-K5,8,12G*)
MAY503	Same as MAY430, except pMX503 (*hht2-Δ430, hhf2-K5,8,12,16G*)
MAY504	Same as MAY430, except pMX504 (*hht2-Δ430, hhf2-K8, 12, 16G*)
MAY505	Same as MAY430, except pMX505 (*hht2-Δ430, hhf2-K5,12,16G*)
MAY506	Same as MAY430, except pMX506 (*hht2-Δ430, hhf2-K5,8,16G*)
MAY512G	Same as RMY430, except pK512G (*hht2-Δ430, hhf2-K5, 12G*)
MAY512R	Same as RMY430, except pK512G (*hht2-Δ430, hhf2-K5, 12R*)
MAY58R	Same as RMY430, except pK58R (*hh2-Δ430, hhf2-K5, 8R*)

phan, lysine, and uracil in various combinations and containing either galactose (SG) or glucose (SD) were prepared as described (13). Yeast transformation was carried out as reported (20).

Plasmid Supercoiling *in Vivo*. Total yeast DNA was isolated from cells grown as indicated in *Results*, and the topology of the endogenous 2-μ plasmid was analyzed by electrophoresing 10 μg of total cellular DNA through 0.8% agarose gels with 10 μg/ml chloroquine. At this chloroquine concentration the topoisomers that are resolved are positively supercoiled. The electrophoresed DNA was subjected to Southern blot analysis using a ^{32}P-labeled *Eco*RI fragment, from plasmid Yep24, that contains 2-μ DNA sequence (12).

Plasmid Supercoiling *in Vitro*. Yeast cells were grown in galactose to mid-logarithmic phase and then shifted to glucose-containing medium for 12 h to deplete wild-type H3 and H4 expressed from plasmid A (Fig. 1). Whole cell extracts (WCEs), containing histones and necessary components for replication-independent nucleosome assembly, were prepared from these cells. Each WCE used in the nucleosome assembly reaction (12, 21) contained 100 μg of protein. At various times of incubation, plasmid DNA was purified, and topoisomers were resolved on nondenaturing 1% agarose gels. The gel was dried and the intensities of bands were quantitated by PhosphorImager (Molecular Dynamics) analysis.

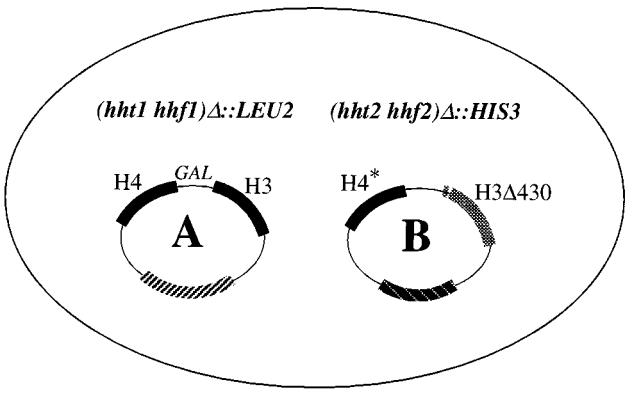

FIG. 1. Scheme for genetic analysis of histone H3 and H4 mutants. Histone H4 mutations in combination with the histone H3 N-terminal deletion Δ4–30 were analyzed by shifting cultures from galactose- to glucose-containing media. By shutting off wild-type H3 and H4 synthesis under control of the *GAL1–10* promoter (in plasmid A) it is possible to examine the effects of mutated H3 and H4 (produced from plasmid B) on cell growth and nucleosome assembly in glucose medium. * indicates the various H4 mutations.

RESULTS

Histone H4 K5 and K12 Are Not Required for Cell Viability. While the deletion of either the H3 or H4 N terminus allows cell growth and nucleosome assembly, the absence of both of these N termini in the same cell prevents viability and assembly (10–12). Because of this redundancy in function it was essential that we examine mutations of histone H4 acetylation sites K5, K8, K12, and K16 in the absence of the H3 N terminus. H3 N-terminal residues 4–30, which contain the five lysines (K9, K14, K18, K23, and K27) acetylated in mammals and which are conserved in yeast, were deleted. These mutations were made in strain RMY430 (17) in which both chromosomal copies of the genes for histones H3 and H4 (*HHT1-HHF1* and *HHT2-HHF2*) are deleted. RMY430 carries an episomal wild-type H4 gene (*HHF2*) and an H3 gene (*hht2-Δ430*), deleted for the region encoding residues 4–30 (plasmid B in Fig. 1). Derivatives of this strain were then constructed by introducing various mutations in the H4 gene on plasmid B. To test for viability of the various H4 mutations when combined with H3 Δ4–30, a plasmid (pRM102) was introduced in which wild-type H3 and H4 genes (*HHT2* and *HHF2*) are under control of the *GAL1–10* promoters (plasmid A). These strains were maintained in galactose-containing medium to express both wild-type H3 and H4 from plasmid A. Shifting cultures of these strains to glucose-containing medium represses the *GAL1–10* promoter in plasmid A, allowing the phenotypes of the various H3 and H4 mutant combinations to be analyzed. Lethality was indicated when a strain failed to grow on glucose medium. To eliminate the possibility that viability of certain H3/H4 double mutants resulted from leaky expression of the *GAL*-controlled wild-type H3 and H4 genes from plasmid A in glucose medium, these strains were further tested for their ability to lose the inherently unstable plasmid A by selecting for loss of its *URA3* marker by using resistance in the presence of 5-fluoroorotic acid (5-FOA) (22). Growth in the absence of plasmid A indicates that the yeast cells rely only on the mutant histone in question for cell viability and nucleosome assembly.

The deletion of H4 residues 4–14 (containing K5, K8, and K12) was lethal with H3 Δ4–30 (MAY817) (Table 2). Therefore, to determine whether the lysines whose acetylation is correlated with deposition are essential for growth, we mutated K5 and K12 to glycines (K5,12G in strain MAY512G) because these residues are uncharged (as are acetylated lysine residues) and the H4 N terminus is already rich in glycines. They were also mutated to arginines (K5,12R in strain MAY512R) (Table 2) to simulate the charged, unacetylated state. Surprisingly, both strains (MAY512G and MAY512R)

Table 2. Viability of H3/H4 mutants

Strain	H4 mutation	Plus H3 Δ4–30
MAY817	Δ4–14	Lethal
MAY818	Δ4–19	Lethal
MAY806	Δ4–23	Lethal
MAY512G	K5,12, to G	Viable
MAY512R	K5,12, to R	Viable
MAY502	K5,8,12 to G	Lethal
MAY503	K5,8,12,16 to G	Lethal
MAY504	K8,12,16 to G	Viable
MAY505	K5,12,16 to G	Viable
MAY506	K5,8,16 to G	Viable

were viable in glucose and able to lose plasmid A on 5-fluoroorotic acid.

To determine which sites of H4 acetylation were required for cell viability, we constructed triple and quadruple mutations at the four sites of acetylation. In these cases, we focused our efforts on glycine mutations because substitution of four arginines at K5, K8, K12, and K16 strongly inhibits transcription and growth, possibly because of irreversible histone–DNA interactions (14). The strains carrying the K to G mutations at the first three sites of acetylation K5,8,12G (MAY502) or the quadruple mutation K5,8,12,16G (MAY503) were not viable. In contrast, strains carrying similar substitutions K8,12,16G (MAY504) or K5,12,16G (MAY505) or K5,8,16G (MAY506) were viable. These data demonstrate that lethality results only from the simultaneous mutation of all three lysines, K5, K8, and K12. These data also emphasize the difference between the first three lysines and K16. A single lysine residue at site 5 or 8 or 12 in the absence of other acetylatable lysines in H3 and H4 allows cellular viability (MAY504–506) but a lysine at site 16 only (MAY502) results in lethality.

H4 Residues K5 and K12 Are Not Required for Nucleosome Assembly *in Vivo*. We then wished to determine whether H4 K5 and K12 are required for nucleosome assembly *in vivo*. Because formation of one nucleosome introduces one negative superhelical turn in a closed circular DNA molecule (23, 24) the superhelical density of plasmids has been used to assay for nucleosome formation *in vivo* (25, 26). The superhelicity of the 2-μ plasmid endogenous in our strains was measured in galactose (G), in which both wild-type and mutant histones are expressed, and after 4 hr in glucose (D), when only mutant histones are expressed from plasmid B. In agreement with earlier results (12), the H3 Δ4–30 deletion alone caused no obvious change in superhelicity of the 2-μ plasmid when the cells grown in galactose were shifted to glucose (Fig. 2*A*, lanes 1 and 2). Nor did mutation of K5/K12 to glycines or arginines in combination with H3 Δ4–30 (Fig. 2*A*, lanes 3–6).

In contrast, mutations K8,12,16G, K5,12,16G, or K5,8,16G caused some decrease in superhelicity (≈1–1.5 linking number change) (Fig. 2*B*, lanes 7–12), whereas the H4 mutations K5,8,12G (Fig. 2*B*, lanes 3 and 4) and K5,8,12,16G (Fig. 2*B*, lanes 5 and 6) decreased superhelical density more strongly, resulting in a decrease in linking number by ≈3.5–4.0. Given the size of 2-μ plasmid (6.3 kb) and the length of yeast nucleosomal DNA plus linker (≈165 bp) (27), a fully assembled 2-μ plasmid should contain about 38 nucleosomes. Loss of 4 nucleosomes should represent a loss of 10.5% of the normal set of nucleosomes. Although we cannot exclude the possibility that the change in superhelical density is caused by a differential interaction of the histone N termini with DNA, these data argue that the greatest effect on histone octamer/DNA interactions is caused by those mutations that also prevent cellular viability. Interestingly, the decreased superhelical density observed for the mutation K5,8,12,16G is not as great as that in which histone H4 is depleted (25) or in which the H3 and H4 N termini are both deleted simultaneously (12). Therefore, assembly is decreased but is not prevented completely even by substitution of all four sites of H4 acetylation in the complete absence of the H3 N terminus.

The extent of decreased superhelical density observed in the lethal mutants described here is unlikely to result from the lethality of the strains under nonpermissive conditions or from the preferential arrest of these strains at G_2/M (data not

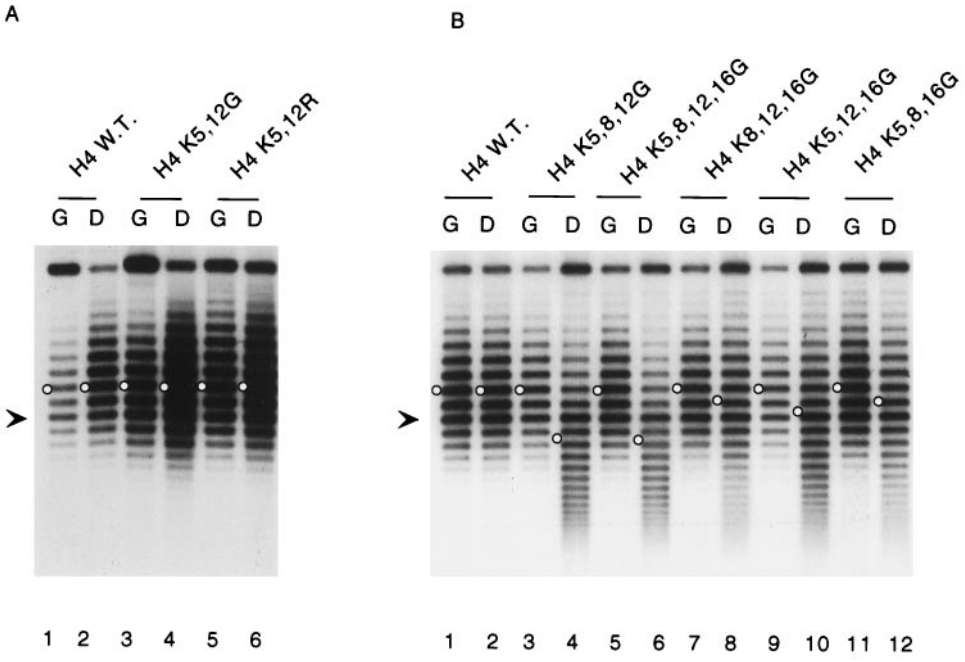

FIG. 2. Superhelical density of endogenous 2-μ plasmid in various histone mutants. The labels at the top indicate various H4 mutations carried on plasmid B in combination with H3 Δ4–30. W.T., wild type. A small open circle on the left of each lane marks the center of distribution of topoisomers identified by densitometric tracing. G and D designate samples from cells grown in galactose and glucose, respectively. These experiments demonstrate that mutation of H4 K5/K12 has no apparent effect on superhelical density of plasmid *in vivo*. The greatest effect on superhelical density, a measure of nucleosome assembly, occurs when K5, K8, and K12 are mutated simultaneously.

shown). We have examined other lethal mutations with similar cell cycle phenotypes (e.g., *cdc14* or *clb1, -2, -3,* and *-4*) and found that they cause no detectable change in plasmid superhelicity when arrested in G_2/M (ref. 28; data not shown).

H4 Residues K5 and K12 Are Not Required for Nucleosome Assembly *in Vitro*. The decrease in plasmid superhelical density *in vivo* described above could be caused by a defect in the pathway leading to assembly rather than by a defect in assembly itself (e.g., decreased histone transport to the nucleus). Moreover, unknown redundant pathways in living yeast cells may compensate for assembly defects. Therefore, we examined the ability of mutant histones to support nucleosome assembly *in vitro* with cellular extracts. Cells grown in galactose were shifted to glucose for 12 h to deplete wild-type H3 and H4 expressed from plasmid A (Fig. 1), allowing the analysis of the effects of H3 Δ4–30 and the wild-type or mutant H4. Whole cell extracts were prepared from these cells and incubated with a relaxed, internally labeled plasmid. Topology of the plasmid after incubation with the extracts was analyzed by agarose gel electrophoresis (Fig. 3).

Extracts from MAY430 containing wild-type H4 and a deletion of the H3 N terminus supported relatively efficient nucleosome assembly as judged by the conversion of relaxed input plasmid (P) DNA (Fig. 3*A*, lane 1) to a supercoiled form (S) (Fig. 3*A*, lane 2). O and R refer to open (nicked) plasmid or the topologically equivalent (relaxed) closed-circular species. The same result was obtained when the H4 N terminus (residues 4–28) was deleted in the presence of the wild-type H3 N terminus (12). Therefore, the H4 or H3 N termini can support *in vitro* assembly in a redundant manner as described earlier (12). Moreover, the severely truncated H3 and H4 proteins in these strains must be stable enough in these extracts to allow efficient nucleosome assembly.

We first asked whether K5 and K12 were required for assembling H4 in cellular extracts. We found that the extract from cells containing H4 K5,12G assembles nucleosomes *in vitro* in a manner indistinguishable from that with extract from cells containing the wild-type H4 N terminus (Fig. 3*A*; compare lanes 2 and 4). In comparison, extracts from cells carrying H4 K5,8,12G were severely defective in supporting nucleosome assembly *in vitro,* showing the presence of mostly intermediate species that were not fully supercoiled (Fig. 3*A*, lane 3). Therefore, the first three sites of acetylation may function in a redundant manner to allow nucleosome assembly *in vitro*.

To determine whether certain of these sites or K16 can support nucleosome assembly *in vitro* in the absence of the other sites of acetylation we also examined K8,12,16G, K5,8,16G, and K5,12,16G mutations. We found that, in comparison to K5,8,12G or the quadruple mutation K5,8,12,16G (Fig. 3*B,* lanes 3 and 4) the other triple mutants assembled a much larger fraction of plasmid as supercoiled DNA (Fig. 3*B*, lanes 5–7). While these mutations do cause some decrease in nucleosome assembly judging by the generation of more intermediate species in their presence (Fig. 3*B,* compare lanes 5–7 to lane 2), K5, K8, and K12 are clearly redundant in supporting histone deposition. K16 alone is much less able to support assembly.

To rule out the possibility that some of the extracts were defective in the nucleosome assembly reaction because the cells were losing the necessary assembly factors during the nonviable, glucose arrest period, we added back wild-type histones in control experiments to an extract from a strain in which the H3 and H4 N termini were both deleted and from which extract was obtained after growth arrest in glucose. This addition restored nucleosome assembly to the wild-type level (Fig. 3*C*). Therefore, inviability and glucose arrest do not cause assembly factors to become nonfunctional. Moreover, we have examined histone deposition by using extracts from more than 80 different temperature-sensitive lethal strains at the nonpermissive temperature. None of these showed a defect in

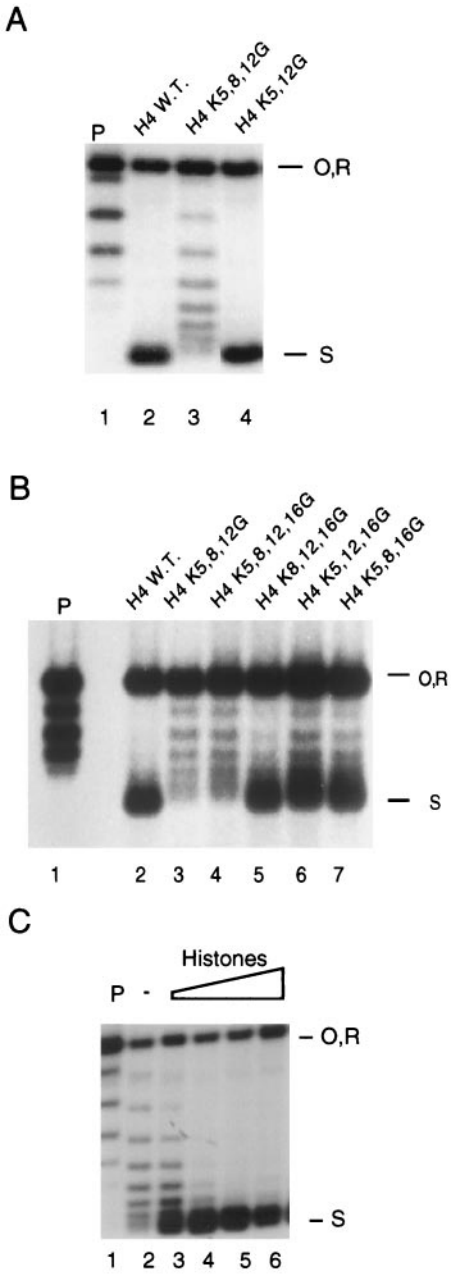

FIG. 3. Nucleosome assembly *in vitro*. Strains were grown in galactose to logarithmic phase, and then shifted to glucose-containing medium for 12 h to repress expression of wild-type H3 and H4 from plasmid B. Whole cell extracts were prepared from these cells and used for the *in vitro* nucleosome assembly reaction. The topology of a closed, relaxed, internally labeled plasmid DNA before (P; lane 1, each panel) and after incubation with extracts was analyzed in agarose gels. Plasmid supercoiling was measured after a 2-min assembly reaction. O, R, and S represent open, relaxed, and supercoiled plasmid DNA, respectively. (*A*) K5/K12 mutagenesis has no evident effect on nucleosome assembly *in vitro* (compare lane 4 to lane 2). In contrast, K5/K8/K12 mutagenesis severely disrupts nucleosome assembly (lane 3). (*B*) Redundancy of H4 K5, K8, and K12 in supporting nucleosome assembly *in vitro*. A comparison of the effects of mutagenesis of K5/K8/K12 (lane 3) and K5/K8/K12/K16 (lane 4) with mutagenesis of K8/K12/K16 (lane 5), K5/K12/K16 (lane 6), and K5/K8/K16 (lane 7). In comparison with wild-type H4 extracts (lane 2), the greatest effects on nucleosome assembly are seen when K5/K8/K12 (or K5/K8/K12/K16) are mutagenized simultaneously (lanes 3 and 4). (*C*) Extract from a mutant strain lacking H3 and H4 N termini can assemble plasmid when wild-type histones are added back to the assembly-defective extract. Wild-type histones (0, 1, 2, 4, or 6 μg in lanes 2–6) were added to 50 μg of extract. Reactions were performed for 60 min at 30°C.

assembly. We also examined strains arrested at G_1/S to G_2/M in the cell cycle because of mutations in cell division cycle proteins (cdc28-1, cdc4-1, cdc7-1, cdc17-1, cdc15-1, cdc15-2, and cdc20-1). None of these mutations decreased the level of assembly in our assays (data not shown). Therefore, the assembly defects we observe with extracts from the nonviable histone mutants are highly unlikely to result from cellular inviability or cell cycle arrest and are likely to be due to the mutant histones themselves. In conclusion, our data demonstrate that K5/K12 (and by inference, K5/K12 diacetylation) cannot be required for nucleosome assembly *in vitro*. In contrast, H4 sites K5 or K8 or K12 (but not K16) can support nucleosome assembly *in vitro* in a redundant manner.

DISCUSSION

Newly synthesized histone H4 is diacetylated (at sites K5/K12) in many eukaryotes. This particular modification pattern is often referred to as the deposition pattern for H4 assembly into nucleosomes (4) and differs from that in mature chromatin, in which H4 is modified by mono-, di-, tri-, and even tetra-acetylation at sites K5, -8, -12, -16. Surprisingly, however, H4 associated with human CAF-1 is not uniquely diacetylated. Instead it is heterogeneously acetylated at sites K5, -8, and -12 (9). Using mutations in histone H4, we find that K5/K12 and by inference, K5/K12 diacetylation, are not required for cell viability and have no obvious effect on nucleosome assembly either *in vivo* or *in vitro*. Instead, the three residues K5, -8, and -12 are required in a highly redundant manner; nucleosome assembly is strongly impaired only when these three lysines are mutated simultaneously. These data argue that acetylation of one or more sites at K5, K8, and K12 allows H4 assembly.

These results help clarify the acetylation state of H3 and H4 associated with human CAF-1 (9). In this complex, H4 is not acetylated in a unique pattern; instead, mono-, di-, and tri-acetylated species were found to be acetylated at K5, K8, and K12, and some H4 (33%) is not acetylated at all (9). A large fraction (60%) of H3 associated with CAF-1 is not acetylated, whereas the rest is to a large extent monoacetylated. These findings can be explained if we assume that, as in yeast, human H4 need only be acetylated at any of the sites K5, -8, or 12 (or a combination of these sites) to be assembled. Moreover, redundancy of the H3 and H4 N-terminal functions in assembly (12) would make it possible for unacetylated H3 to be assembled when ''piggy-backed'' onto acetylated H4. Conversely, the H3 N terminus may mediate H3/H4 assembly in the absence of the H4 N terminus or its acetylation (12).

Our conclusions contrast with those of a recent study in which mutated H3 and H4 mRNAs were microinjected into *Xenopus* embryos to determine their competence for assembly into chromatin. Deletions at the H4 N terminus were tested for assembly in the presence of wild-type H3, and *vice versa*. This work concluded that the sites of acetylation in H3 and H4 are not essential for nucleosome assembly in the *Xenopus* system (29). However, this study did not take into account the redundancy of the H3 and H4 N termini with regards to nucleosome assembly (10, 12). Without examining H4 N-terminal mutations in the absence of the H3 tail it cannot be concluded that histone acetylation is unimportant for nucleosome assembly in either yeast (10, 12) or *Xenopus*.

Our data show that there is a distinction *in vivo* between the function of H4 sites K5, K8, and K12 as opposed to K16. Acetylation at K5/K8/K12 could represent a chemical tag or cause a conformational change in the H4 tail allowing its recognition by chromatin assembly factors. However, it must be kept in mind that the factors that assemble general chromatin *in vivo* in yeast or human cells are not yet known. The yeast CAF-1 complex is not essential for viability or for nucleosome assembly *in vivo* (30–32), arguing for the presence of other pathways that may be important or redundant. The same may be true for the human CAF-1 complex, which has been shown to function in assembly *in vitro* only. The p48 protein, a component of human CAF-1, does interact directly *in vitro* with H4, and it does not require the acetylatable lysines between residues 1–14 for this interaction (33). However, CAF-1 does assemble newly synthesized H3 and H4 but not mature chromosomal histones (7). These are acetylated differently from mature histones (9). Therefore, by these criteria, it would appear that CAF-1 prefers to interact with and assemble uniquely acetylated H3/H4. Perhaps the H4 lysine residues when acetylated affect binding of p48 to H4. Alternatively, other chromatin assembly proteins may interact directly with the acetylated lysine residues.

Why might there be a distinction in function between the first three sites of H4 acetylation and K16? An explanation may lie in the differential requirement for K16 in heterochromatin as opposed to euchromatin. In yeast, the silencing information regulator SIR3 which is present at the telomeres and silent (HM) mating loci interacts with the histone H4 N terminus to help assemble heterochromatin. The H4 residues interacting with SIR3 to repress heterochromatin are residues 16–29 (16, 34, 35), and mutagenesis and cross-linking experiments argue that acetylation of K16 may prevent H4–SIR3 interaction in heterochromatin (34). Assembly of H4 acetylated at K16 would very likely result in altered telomere structure and sterility. Because mutagenesis of H4 K5, K8, and K12 does not cause these effects (34), it is possible that these sites are utilized preferentially in H4 assembly.

Interestingly, in the recent x-ray crystal structure of the nucleosome (36) a region similar to that binding SIR3 (H4 residues 16–25) has been shown to extend from the nucleosome to interact with a highly acidic pocket of the H2A–H2B dimer of an adjacent nucleosome. This interaction may serve to bind two nucleosomes together. The most abundant, monoacetylated form of H4 is acetylated exclusively at K16 in yeast and other eukaryotes (37). Perhaps K16 acetylation helps prevent nucleosome–nucleosome interactions in euchromatin, stimulating transcription, while also preventing promiscuous binding of H4 to SIR3 in euchromatin. These considerations argue that acetylation of K16 may function as an on–off switch, regulating protein–protein interactions.

We need to stress that although the K5/K12 deposition pattern is not uniquely required for deposition, this pattern of acetylation certainly would allow H4 assembly. One enzyme in yeast that may help mediate K5/K12 acetylation is HAT1, a cytoplasmic B-type histone acetyltransferase (38, 39). *hat1* mutations do not have an apparent phenotype (38, 39), so it is possible that there also exist redundant cytoplasmic histone acetyltransferase activities that target other H4 sites. Whether K5/K12 diacetylation allows somewhat more efficient assembly, is a pattern that remains to be altered prior to assembly, or is the indirect result of evolutionary pressures on other factors such as HAT1 remains to be determined.

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