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Regulation of NuA4 Histone Acetyltransferase Activity in Transcription and DNA Repair by Phosphorylation of Histone H4

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The NuA4 complex is a histone H4/H2A acetyltransferase involved in transcription and DNA repair. While histone acetylation is important in many processes, it has become increasingly clear that additional histone modifications also play a crucial interrelated role. To understand how NuA4 action is regulated, we tested various H4 tail peptides harboring known modifications in HAT assays. While dimethylation at arginine 3 (R3M) had little effect on NuA4 activity, phosphorylation of serine 1 (S1P) strongly decreased the ability of the complex to acetylate H4 peptides. However, R3M in combination with S1P alleviates the repression of NuA4 activity. Chromatin from cells treated with DNA damage-inducing agents shows an increase in phosphorylation of serine 1 and a concomitant decrease in H4 acetylation. We found that casein kinase 2 phosphorylates histone H4 and associates with the Rpd3 deacetylase complex, demonstrating a physical connection between phosphorylation of serine 1 and unacetylated H4 tails. Chromatin immunoprecipitation experiments also link local phosphorylation of H4 with its deacetylation, during both transcription and DNA repair. Time course chromatin immunoprecipitation data support a model in which histone H4 phosphorylation occurs after NuA4 action during double-strand break repair at the step of chromatin restoration and deacetylation. These findings demonstrate that H4 phospho-serine 1 regulates chromatin acetylation by the NuA4 complex and that this process is important for normal gene expression and DNA repair.

Posttranslational modifications of histone proteins in chromatin are a critical means of regulation for DNA-related processes in the cell. It has long been known that posttranslational modifications, such as acetylation, methylation, phosphorylation, and ubiquitination of histones, exist in the cell. While historically histone acetylation was the most widely studied for its correlation with active transcription, recent years have seen the emergence of roles for histone methylation, phosphorylation, and ubiquitination. It has become increasingly clear that each of these modifications or marks is intimately involved in the regulation of various cellular processes (52). Addition or removal of acetylation or phosphorylation alters the charge of their target residues, which may physically affect the interaction of histone tails with DNA, providing a more "open or closed" state. Alternatively, any of these marks might provide recognition sites for protein modules, as shown for the bromodomains of TAF1 ($TAF_{II}250$) and Bdf1, which were shown to preferentially bind to multiply acetylated forms of histone H4, or the chromodomains of HP1 and Polycomb, which specifically bind to methylated lysine 9 or 27 on histone H3, respectively (18, 26, 31, 38).

The histone N-terminal tails contain an extraordinarily high concentration of known and putative modification sites. To date, the most extensively studied region of histone modification is found in the histone H3 N-terminal tail. Phosphorylation of serine 10 of histone H3 has been associated with both mitotic condensation and gene activation by mitogenic factors. Interestingly, H3 peptides containing phospho-serine 10 potentiate acetylation of neighboring lysine 14 by several histone acetyltransferases (HATs), including Gcn5 (6, 35). However, this stimulation could not be reproduced in HAT assays using chromatin substrates and native SAGA complex (48). Nevertheless, this combination of two marks is associated with certain actively transcribed genes, including the immediate-early genes upon epidermal growth factor stimulation and also a subset of Gcn5-dependent genes in yeast (6, 35).

As briefly mentioned above, methylation of lysine 9 on histone H3 is a docking point for HP1, a protein sufficient to nucleate repressive heterochromatin. Thus, it is interesting that methylation of lysine 9 is also refractory to phosphorylation of serine 10 (17). It is evident from these studies that neighboring histone modifications can directly affect one another (16, 17). Interestingly, this type of dynamic interplay can also be seen in *trans*. The ubiquitination of lysine 123 on the C terminus of histone H2B by Rad6 is required for the subsequent methylation of lysine 4 and lysine 79 on histone H3 (57), though the mechanism remains unknown.

While most nuclear HATs target mainly nucleosomal histone H3, the NuA4 histone acetyltransferase complex acetylates lysine residues in the histone H4 tail (as well as H2A) (14). Little is known about the interplay of modifications on the histone H4 tail. We therefore set out to determine how the various modifications in the H4 tail affect the ability of NuA4

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to acetylate. Here, we show that phosphorylation of serine 1 decreases the HAT function of NuA4 on H4 tail peptides. However, if arginine 3 is also dimethylated (R3M), NuA4 acetylation is recovered to levels seen on the unmodified H4 tail. Interestingly, S1 phosphorylation (S1P) is regulated in yeast. Under DNA damage conditions with increased levels of phospho-serine 1 (P-Ser1), there is a drop in acetylation of histone H4. However, blocking the cells in S phase also increases phosphorylation of serine 1 with no apparent loss of acetylation. Notably, casein kinase 2 (CK2), a DNA damage-regulated kinase, can phosphorylate histone H4 at serine 1 and is found associated with the Sin3/Rpd3 histone deacetylase complex, thus connecting phospho-serine 1 with loss of acetylation on histone H4. This is confirmed in vivo, as local increase of H4 phosphorylation correlates with its deacetylation during both gene transcription and DNA repair.

MATERIALS AND METHODS

Peptides. Synthetic peptides were synthesized corresponding to the N-terminal 19 or 25 residues of histone H4 with or without the following modifications: phospho-serine 1, dimethyl-arginine 3, and dimethyl-lysine 20. Peptides multiply acetylated at lysine 5, 8, 12, and/or 16 (25-mers) were a generous gift from Patrick Grant. Doubly and singly modified peptide combinations (22-mers) with phospho-serine 1, dimethyl-arginine 3, and acetyl-lysine 5 were kindly provided by C. D. Allis. All H4 peptides were N-terminally acetylated, as found in vivo, and verified by mass spectrometry after synthesis/purification to confirm the presence of the specific modifications.

Protein purification and HAT, phosphatase, and kinase assays. Recombinant Esa1 was purified as described previously (15). Purified NuA4 was obtained either by conventional purification of yeast extracts over nickel agarose, mono Q, and superose 6 columns as described previously (1) or by tandem affinity purification (TAP) of tagged-Epl1 (5). HAT assays were performed as described previously (1). For peptide experiments, 300 ng of unmodified and various modified histone H4 N-terminal tail peptides were used as substrates. After liquid scintillation counting, values were normalized compared to unmodified peptide and are presented in bar graph form. Relative peptide amounts were verified by optical density (OD) and staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All HAT assay experiments were repeated several times with similar results. The data presented are from a single experiment done in triplicate. For phosphatase assays, 300 ng phospho-serine 1 or unmodified peptide was incubated with 200 units of lambda phosphatase (New England Biotechnology) in conditions similar to HAT assays (50 mM Tris-HCl, pH 8, 5% glycerol, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol [DTT]) with 2 mM $MnCl_2$ for 30 min at 30°C. In some cases, either prior to or after phosphatase, 0.5 mM sodium vanadate, a phosphatase inhibitor, was included in the reaction mixture. HAT reactions with Esa1 or NuA4 were performed in final conditions of 50 mM NaCl, 50 mM Tris-HCl, pH 8, 5% glycerol, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 1.3 mM $MnCl_2$. For kinase assays, reactions were carried out with 2 μg of histones (purified or recombinant) in final conditions of 80 mM NaCl/KCl, 25 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 1 mM DTT, 50 μM cold ATP, and 1 μCi [γ-^{32}P]ATP for 30 min at 30°C. Samples were run on 18% SDS-PAGE, blotted to nitrocellulose, dried, and exposed to film. For Western blots, assays were performed similarly except that the membranes were probed with anti-S1P antibody as described above. Sin3, Rpd3, and Ckb2 were purified by tandem affinity using TAP-tagged strains (Euroscarf) (20) as described previously (30).

Yeast chromatin preparation and Western blot and dot blot analyses. Cells were grown in yeast-peptone-dextrose (YPD) until they reached an OD at 600 nm (OD_{600}) of 1 to 1.2, at which point alpha factor (1 μM), nocodozole (10 μg/ml), hydroxyurea (HU) (200 mM), methyl methane-sulfonate (MMS) (0.05%), rapamycin (25 nM), or nothing (control) was added while growth continued for 2 h. For the stationary-phase condition, cells were stopped at an OD_{600} of 6 to 7. Native yeast chromatin was prepared as described previously (30). Equivalent amounts (2 μg) of purified chromatin were run in 18% SDS-PAGE, blotted to nitrocellulose, and probed overnight at 4°C with the following antihistone antibodies at 1:1,000 dilution unless otherwise indicated. From Upstate Biotechnology, α-phospho-S10 H3, α-dimethyl-K4 H3 (1:5,000), α-acetylated H3, α-dimethyl-R3 H4, α-hyperacetylated H4 (1:3,000) (cross-reacts with acetylated H2A), α-K8Ac H4, α-K12Ac H4, and α-H4 (1:250) were used, while α-K5Ac and α-K16Ac were obtained from Serotec. α-phospho-S1 H4 (1:2,000) was generously provided by C. D. Allis (3). α-phospho-S129 H2A (1:500) was a kind gift from Jessica Downs (13). Ponceau staining of the membrane was used to determine equivalent loading. For the epitope-blocking measurement (see Fig. 4B), 100 ng of histone H4 tail peptides was dot blotted to nitrocellulose and probed with α-S1P, α-R3Me2, and α-K5Ac antibodies at the same dilutions described above. For the kinase assays, purified yeast free histones obtained by acid extraction of partially purified chromatin from wild-type (WT) and H4-S1A mutant cells were used as substrates.

Yeast strains, plasmid mutagenesis, and growth assays. Strain BY4741 from Resgen was used for purification of native chromatin. Yeast strain UCC1111, with both chromosomal copies of histone H3 and H4 deleted and harboring an episomal *HHT2-HHF2* locus, and plasmid pMP3 were kindly provided by D. Gottschling (25). Histone H4 serine 1-to-alanine (S1A) or -glutamate (S1E) and arginine 3-to-alanine (R3A) or -glutamine (R3Q) mutations were introduced into pMP3 (*ARS*/*CEN*/*TRP1*), a plasmid containing the *HHT2-HHF2* copy of histones H3 and H4, by PCR mutagenesis using the Stratagene QuickChange protocol. Plasmids were sequenced for the coding regions of both histones H3 and H4 and transformed into UCC1111. In order to aid removal of the WT *ADE2* plasmid, transformants were grown for two cycles to an OD_{600} of at least 2.5 in synthetic dropout (SD) medium lacking tryptophan with 0.4 μg/ml adenine and plated on selective medium (SD Trp^-), generating strains QY829 (WT), QY830 (S1A), QY831 (S1E), QY832 (R3A), and QY8733 (R3Q). For growth analysis, the WT and strains with histone H4 mutations were grown for two generations to an OD_{600} of 0.5 to 1 and diluted to an OD_{600} of 0.02, followed by four serial fivefold dilutions. These were plated on YPD, YPD plus 0.03% MMS, and YPD plus 130 mM HU and grown for 3 to 4 days at 30°C.

ChIP assays. For real-time PCR analysis of chromatin immunoprecipitation (ChIP) experiments on the *HSP104* gene, BY4741 cells were grown in YPD at 30°C until log phase and shifted to 39°C for 10 min (changed to prewarmed medium). The ChIP protocol was as described before (39). For the experiment at an HO-induced DNA double-strand break (see Fig. 7), JKM179 cells with an integrated GAL-HO cassette were used (32). Cultures were incubated overnight in rich medium containing raffinose before direct addition of galactose and incubated for the indicated time prior to cross-linking. In these experiments, anti-PS129H2A (3 μl), anti-PS1H4 (3 μl), and anti-AcK8H4 (0.5 μl; Upstate) were used. The primers used in the PCRs (available upon request) were analyzed for linearity range and efficiency using a LightCycler (Roche). All numbers are based on at least duplicate PCRs, and variation was less than 15%. Values were calculated as a ratio of percentage IP at the region surrounding the HO break versus a control locus (a large intergenic region on chromosome V) (39) after subtraction of signal obtained with no antibody. Data are presented as the change in this ratio after HO induction (time zero set to 1). For the experiment at *HSP104*, the data are presented as a direct percentage of IP at the different loci and conditions.

RESULTS

Each conserved lysine residue of the histone H4 tail can be independently acetylated by Esa1 and NuA4. It was previously shown that Esa1 alone and as part of the NuA4 complex can acetylate all four of the acetylatable lysine residues in the H4 tail (1, 8, 40). We confirmed this by testing the abilities of these proteins to acetylate histone H4 tail peptides containing only one available lysine (K5, K8, K12, or K16) or the fully acetylated substrate (H4Ac) compared to the unmodified peptide (H4wt) (Fig. 1A). These peptides were incubated with recombinant Esa1 or purified NuA4 complex and ^{3}H–acetyl-coenzyme A. Filter binding and liquid scintillation counting determined the level of acetylation, and values were normalized to the unmodified peptide (Fig. 1A). Indeed, Esa1 and NuA4 acetylate lysines 5, 8, 12, and 16 individually at similar levels. On the other hand, the sum of each individual acetylation does not reach the acetylation level of the fully available wild-type peptide, arguing that Esa1/NuA4 can acetylate more than one residue during interaction with its substrate. This cooperativity

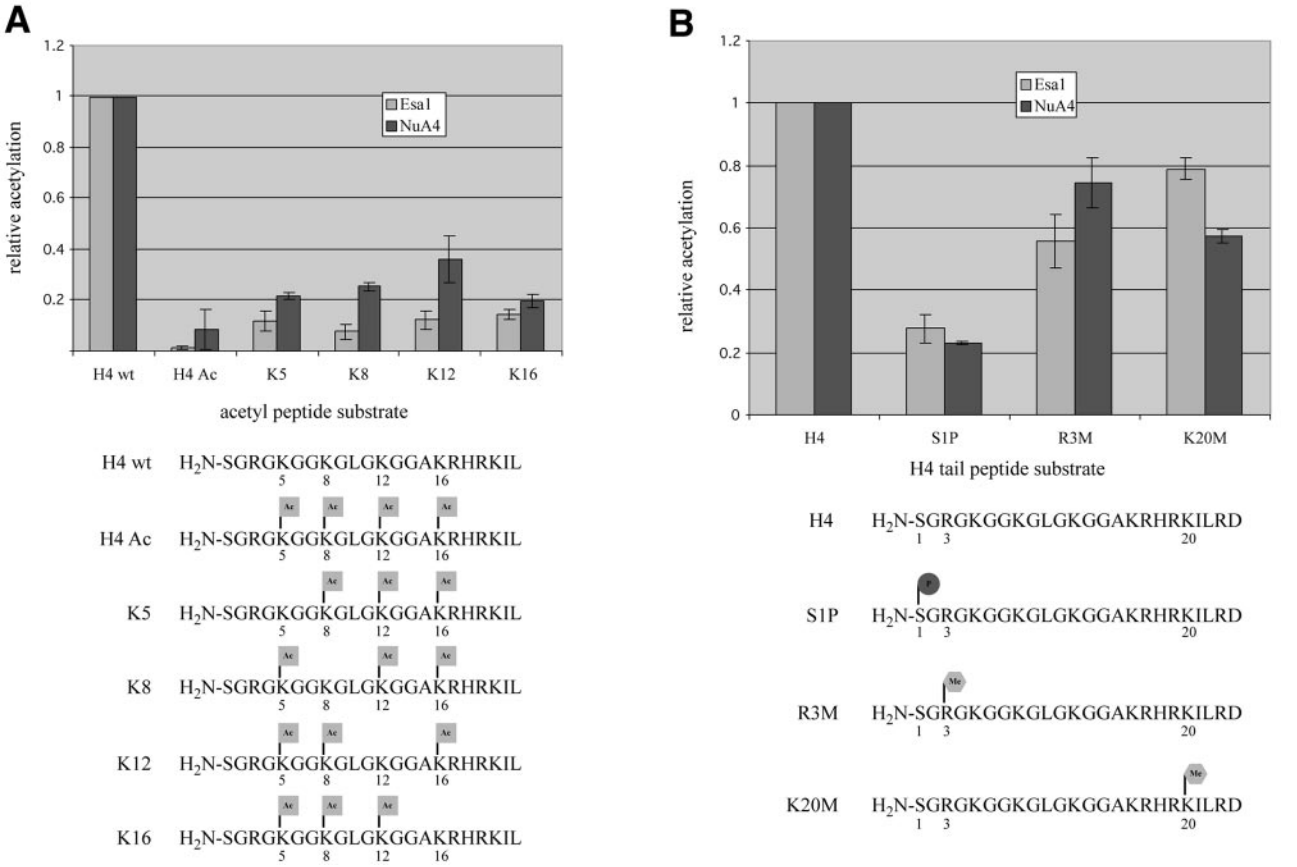

FIG. 1. Phosphorylation of H4 serine 1 inhibits hyperacetylation of its tail by Esa1/NuA4. (A) Esa1 and NuA4 can independently acetylate each lysine residue of the histone H4 tail but prefer to do multiple acetylations. H4 tail peptides were synthesized with or without the acetyl moieties at the conserved lysines 5, 8, 12, and 16 as shown. All peptides are acetylated at the N terminus, as found in vivo. HAT assays were performed with 300 ng of the indicated peptide and either purified NuA4 complex or recombinant Esa1 (catalytic subunit of NuA4), incubated for 30 min at 30°C, spotted on Whatman filters, washed, and subjected to scintillation counting. Acetylation values were normalized to WT H4. The error bars indicate standard deviations. (B) Phosphorylation of serine 1 inhibits acetylation of the H4 N-terminal domain by Esa1 and NuA4. Peptides were synthesized with or without modifications, including phosphorylation of serine 1 (S1P), dimethylation of arginine 3 (R3M), and dimethylation of lysine 20 (K20M). HAT assays were carried out as for panel A.

of lysine acetylation on a single H4 tail by NuA4 was previously suggested by TAU gel analysis of acetylated nucleosomes (1).

Phosphorylation of serine 1 inhibits acetylation of the H4 N-terminal domain by Esa1 and NuA4. The first defined effect of one histone modification on another came from reports showing that histone H3 peptides phosphorylated at serine 10 are better substrates for acetylation of lysine 14 by several HATs, including Gcn5 (6, 9, 35). We wanted to investigate the effect of known histone H4 modifications on the ability of NuA4 to acetylate its target lysines in the histone H4 tail. Synthetic peptides corresponding to the N-terminal residues of histone H4 either without modification (H4) or containing phospho-serine at position 1 (S1P), dimethyl-arginine at position 3 (R3M), or dimethyl-lysine at position 20 (K20M) were subjected to HAT assays with Esa1 or NuA4 as shown in Fig. 1A. While little significant effect was seen with the methylated residues, phospho-serine 1 clearly inhibits the HAT activity of Esa1 and NuA4.

NuA4 activity is recovered upon dephosphorylation of histone H4 at serine 1. To confirm this inhibitory effect of serine 1 phosphorylation, we carried out assays on the unmodified and phosphorylated peptides with lambda phosphatase. The order of addition of reagents is depicted in Fig. 2A, bottom. When the S1P peptide was exposed to phosphatase prior to the action of Esa1 or NuA4, full HAT activity was recovered (Fig. 2A). The results obtained using the phosphatase inhibitor sodium orthovanadate added prior to the HAT reaction demonstrated that the recovery of acetylation was not due to a dephosphorylation event of NuA4. This is also corroborated by the fact that recombinant Esa1 purified from bacteria should not bear any posttranslational modifications and therefore should not be affected by phosphatase treatment. The effectiveness of the sodium orthovanadate was proven by preincubating the phosphatase with inhibitor prior to peptide exposure. Importantly, we determined if this effect was also seen on bulk cellular histones (Fig. 2B). Purified histone proteins were subjected to phosphatase assays, followed by incubation with the NuA4 HAT complex. Interestingly, under conditions where phospho-serine 1 signal was lost from histone H4 by Western blotting (Fig. 2B, bottom), the HAT activity of NuA4 was increased by approximately 45% (Fig. 2B, top), supporting

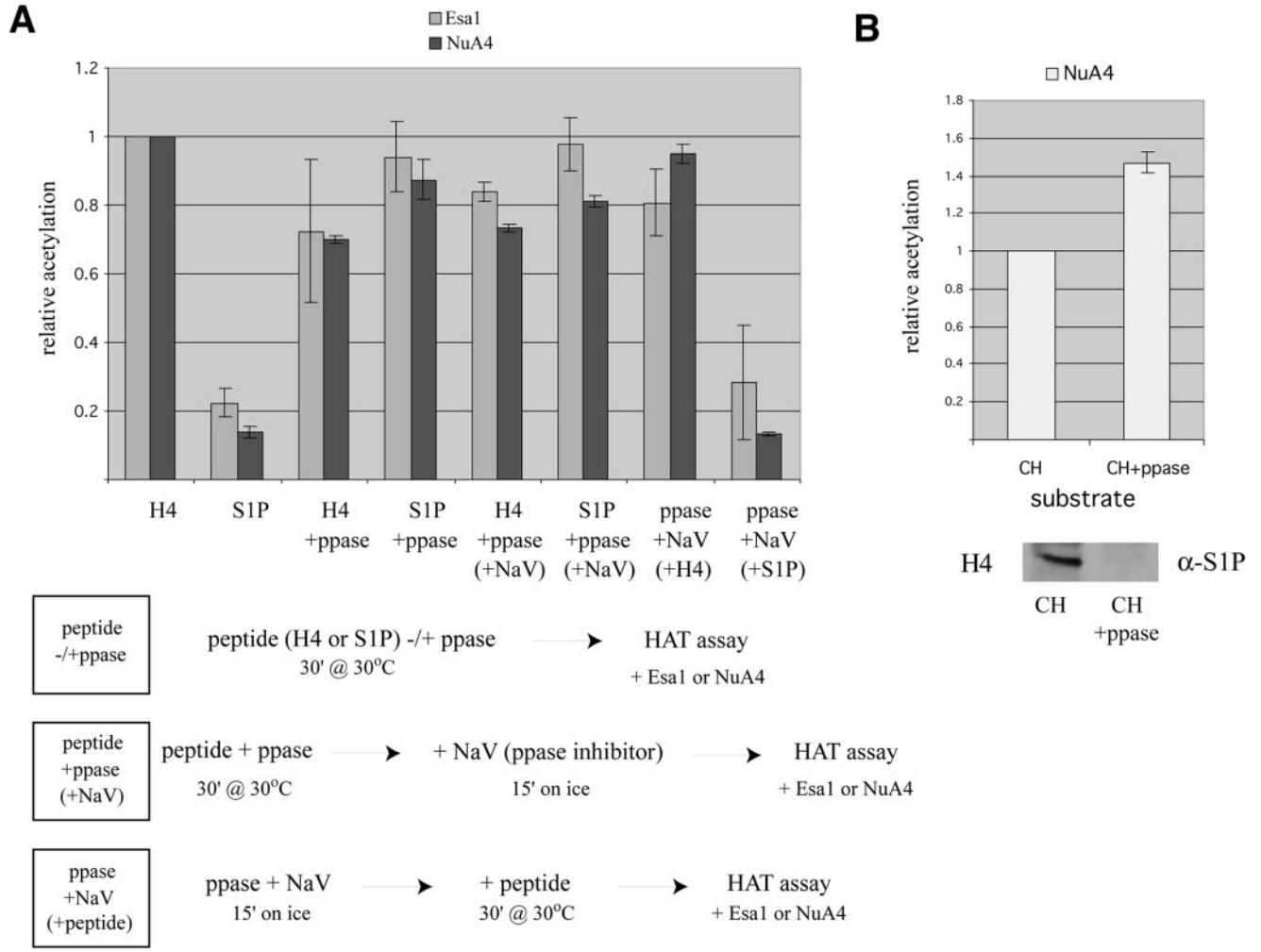

FIG. 2. NuA4 activity is recovered upon dephosphorylation of histone H4 at serine 1. (A) Lambda phosphatase assays were performed on S1P and control H4 peptides as outlined below the graph. When phosphatase (ppase) action occurred, NuA4 activity was restored to levels observed on the previously unphosphorylated peptide (H4). Controls were carried out to show that the inhibitor was effective and not affecting NuA4 under these conditions. NaV, sodium orthovanadate. The error bars indicate standard deviations. (B) A similar phosphatase treatment was performed on 0.5 μg of purified human core histones (CH) in the absence or presence of lambda phosphatase, and they were subjected to HAT assays with NuA4. While Western blotting showed the complete dephosphorylation of histone H4 serine 1, a reproducible 45% increase in NuA4 activity was observed.

the idea that phosphorylation of serine 1 on histone H4 is inhibitory to acetylation by NuA4.

Methylation of histone H4 arginine 3 alleviates the inhibitory effect of phospho-serine 1 on NuA4 activity. In order to assess other possible consequences of multiple modifications on the H4 tail, we used 22-mer peptides containing the single modifications phospho-serine 1 (S1P), dimethyl-arginine 3 (R3M), or acetyl-lysine 5 (K5Ac) or double combinations of these marks. While Esa1 and NuA4 complex again showed decreased activity on phospho-serine 1 and little effect with methyl-arginine 3, they were also inhibited by acetyl-lysine 5 (Fig. 3). The level of the inhibition cannot simply be explained by the loss of this single acetylatable lysine residue (Fig. 1A) and suggests that Esa1 can read an order of lysine acetylation on the H4 tail, as implied by the cooperativity mechanism shown in Fig. 1 (1). However, the most striking result involves the peptide harboring the double modification of S1P and R3M, which exhibits acetylation levels similar to those of the unmodified peptide. We also noted that the inhibition seen with K5Ac is not affected by arginine 3 methylation. Thus, the addition of two methyl groups to arginine 3 abrogates the inhibitory activity of phospho-serine 1 on Esa1/NuA4.

Phosphorylation of histone H4 at serine 1 is regulated in vivo. It was interesting that for histone H4, phosphorylation had a detrimental effect on acetylation by NuA4, whereas histone H3 phospho-serine 10 has been suggested to play a positive role in Gcn5-dependent acetylation (6, 35). In addition, the methylation of arginine 3 negates phospho-serine 1 inhibition. In light of this, we wanted to see if these modifications were regulated during the cell cycle and under certain cellular conditions. Yeast cells were grown to an OD_{600} of 1 to 1.2 and then treated with either alpha factor, which arrests cells in the G_1 phase of the cell cycle; hydroxyurea, which arrests cells in S phase; nocodazole, which prevents the transition

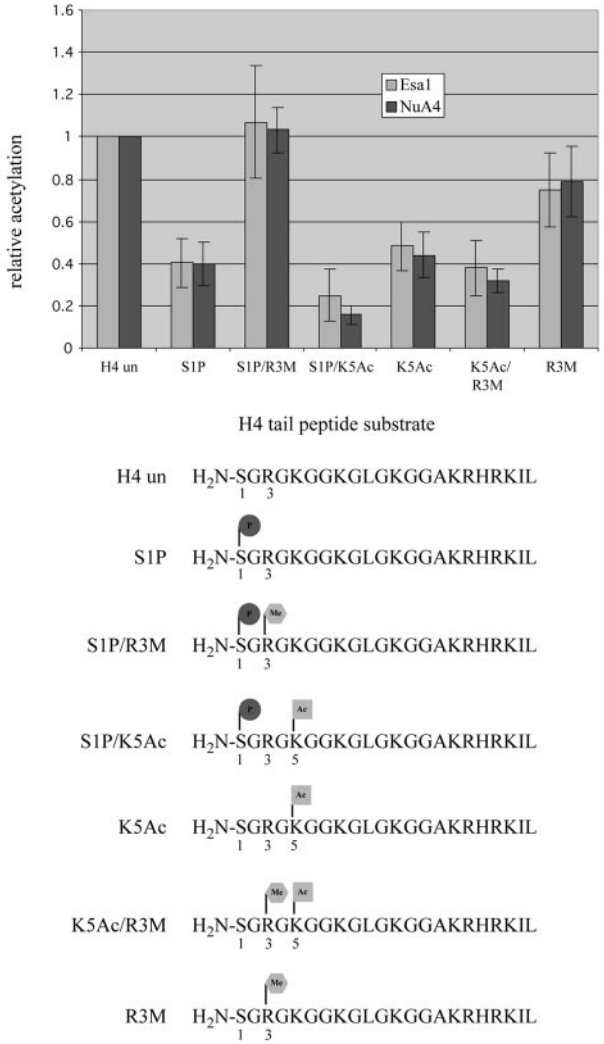

FIG. 3. Methylation of histone H4 arginine 3 alleviates the inhibitory effect of phospho-serine 1 on NuA4 activity. H4 tail peptides were synthesized with various combinations of modifications (S1P, R3M, and K5Ac) as shown. HAT assays were carried out as for Fig. 1. Note that K5Ac is also a strong inhibitor of NuA4 activity, though this effect is not modulated by methylation of arginine 3. The error bars indicate standard deviations.

from G_2 to M phase; MMS, which causes single- and double-strand breaks; rapamycin, which is an inhibitor of the TOR (target of rapamycin) pathway; or nothing (control) for a period of 2 hours. After isolation of nuclei, native chromatin was purified and samples were run in SDS-PAGE and blotted to nitrocellulose. When we assayed for the presence of modified histones by Western blotting, we observed striking variability in the levels of serine 1 phosphorylation, as well as in acetylation of histone H4 (Fig. 4A). Interestingly, we saw increased levels of phospho-serine 1 in both MMS- and hydroxyurea-treated chromatin. As previously mentioned, MMS causes single- and double-strand breaks, and sensitivity to this drug implies a role in DNA repair, while HU blocks DNA replication. In the case of MMS treatment, except for lysine 16, histone H4 acetylation also decreased significantly compared to the control lane,

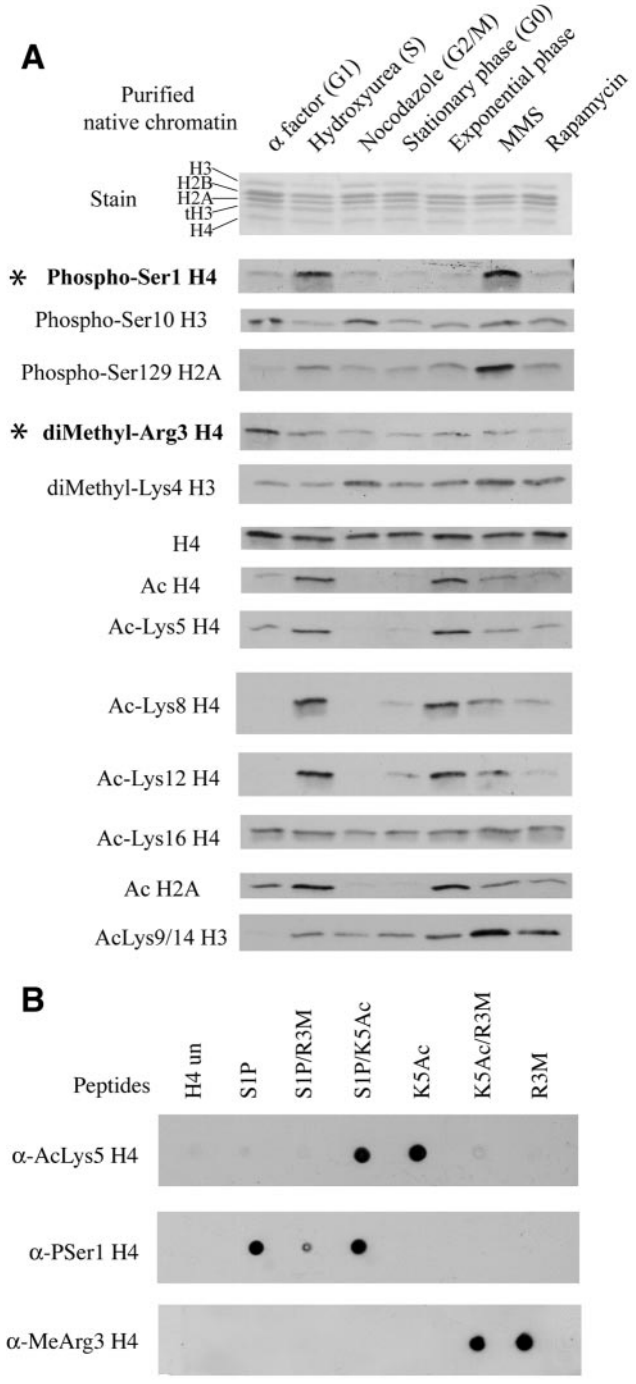

FIG. 4. Phosphorylation of histone H4 at serine 1 is regulated in vivo. (A) Yeast cultures were grown in the absence (control) or presence of the indicated drugs or substances, which induce cell cycle blockage or signaling response. Native chromatin was purified from yeast and analyzed by SDS-PAGE/Western blotting with the indicated antibodies. (B) Dot blots using 100 ng of the indicated peptides were probed with anti-S1P, anti-R3M, or anti-K5Ac antibody to test the effects of neighboring modifications on epitope recognition.

agreeing well with our peptide data showing that phosphorylation of serine 1 is not conducive to acetylation of the same H4 tail. We also noted that phospho-serine 129 of histone H2A was specifically induced under MMS conditions, as previously reported (13).

However, phospho-serine 1 is also stimulated in the presence of hydroxyurea, while a concomitant decrease in histone H4 acetylation is not observed. This does not accord with our theory that phosphorylation of serine 1 is inhibitory to acetylation of the H4 tail. However, H2A phospho-serine 129 is not induced in these conditions, indicating that at least in this case, H4 serine 1 phosphorylation is not a consequence of DNA damage. There was also no apparent increase in dimethylation of arginine 3 in HU-treated cells, which could have explained the apparent simultaneous increase of H4 phosphorylation/acetylation (since R3M counteracts the inhibitory effect of S1P on NuA4-dependent acetylation [Fig. 3]). In light of these data, we questioned the ability of these modification-specific antibodies to recognize their epitopes in the presence of multiple marks on the same histone tail. A dot blot was performed with peptides harboring the single or double modifications shown in Fig. 3 to test the antibodies against S1P, R3M, and K5Ac. As seen in Fig. 4B, each antibody recognized its specific singly modified peptide. While both anti-S1P and anti-K5Ac detect the double S1P/K5Ac peptide, signals are greatly reduced when arginine 3 is dimethylated near their target marks (S1P/R3M and R3M/K5Ac). Similarly, anti-R3M does not recognize the S1P/R3M double modification. These results indicate that multiple modifications on the histone tails may mask neighboring epitopes, resulting in the inhibition of antibody recognition in Western blots and chromatin immunoprecipitations. Thus, the increase of H4 acetylation/phosphorylation that we see in chromatin from cells arrested in S phase may also be associated with an increase of H4 methylation at arginine 3, but we lack the means to detect it.

Mutation of histone H4 at serine 1 does not cause sensitivity to MMS or hydroxyurea. Phosphorylation of histone H2A at serine 129 is induced in MMS, and mutation of this residue causes MMS sensitivity (13). Since an increase in phospho-serine 1 on histone H4 was also brought on during MMS treatment, we wondered if mutation of this residue in yeast cells would also provoke sensitivity to this DNA-damaging agent. Site-directed mutagenesis was performed on a plasmid encoding histones H3 and H4. Mutations at serine 1 and arginine 3 were used to generate strains expressing wild-type histone H3 and histone H4 harboring these altered residues. These cells were then subjected to growth assays on YPD in the absence or presence of MMS or hydroxyurea (Fig. 5A). Similar growth of all mutants compared to the wild-type H4 indicated no sensitivity to either MMS or HU. One important note is that all these strains containing one episomal copy of histones H3 and H4 grow significantly more slowly than those bearing two chromosomal copies (Fig. 5A).

The Rpd3 histone deacetylase is physically linked to a histone H4 serine 1 kinase. If phosphorylation of H4 serine 1 is functionally linked to DNA damage response and blocking nucleosomal H4 acetylation, we would expect the responsible kinase to be regulated by DNA damage and to be linked to histone deacetylation. We tested a large number of kinases involved in DNA damage response, including Mec1, Tel1, Rad9, Dun1, Chk1, Rad53, and Hrr25, but their mutation had no significant effect on the MMS-induced H4 P-Ser1 signal (data not shown). Interestingly, independent yeast proteomic studies showed that affinity-purified Sin3/Rpd3 histone deacetylase complexes were found associated with the casein kinase II tetramer (Cka1/2 and Ckb1/2) (20, 22). Furthermore, CK2 was shown to be involved in the cellular response to DNA

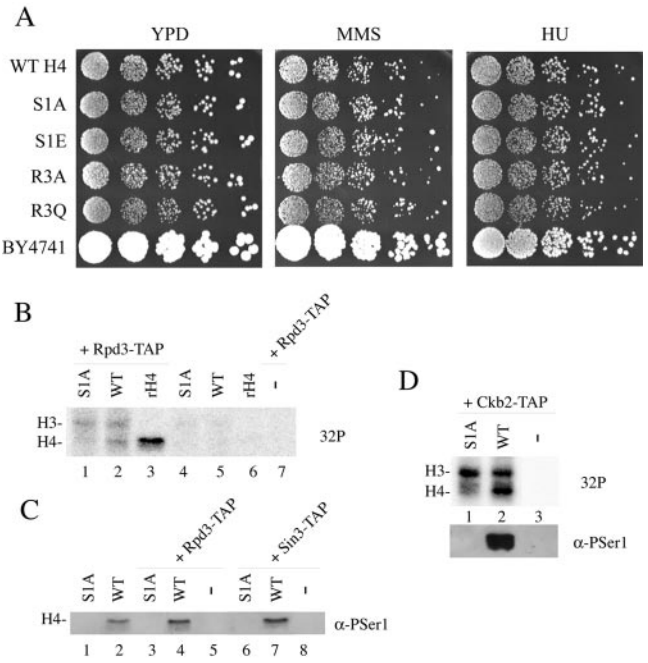

FIG. 5. A histone H4 Ser1 kinase is found associated with the Sin3/Rpd3 histone deacetylase complex. (A) Strains bearing mutations in residue serine 1 or arginine 3 of histone H4 are not sensitive to MMS or hydroxyurea. Wild-type and the indicated mutant histone H4 strains with episomally expressed histone H3 and H4 in addition to normal yeast cells (chromosomally expressed histones; BY4741) were grown to log phase (OD_{600}, 0.5 to 1). Fivefold serial dilutions starting from an OD_{600} of 0.02 were plated on YPD, YPD plus 0.03% MMS, and YPD plus 130 mM HU and grown for 3 to 4 days at 30°C. (B) The Rpd3 complex is associated with histone kinase activities, including one for H4 Ser1. Recombinant yeast histone H4 (rH4; lanes 3 and 6) and purified yeast histones from the strains in panel A (wild type, lanes 2 and 5; S1A, lanes 1 and 4) were incubated in the absence (lanes 4 to 6) and presence (lanes 1 to 3) of affinity-purified Rpd3 complexes with both cold ATP and [γ-^{32}P]ATP, separated by SDS-PAGE, transferred to nitrocellulose, and exposed to film. A control reaction with Rpd3-TAP only is shown in lane 7. Kinase activity was found associated with Rpd3-TAP that targets both recombinant and purified histone H4 (lanes 2 and 3), but not when serine 1 was mutated (lane 1). (C) Reactions similar to those in panel B were performed with purified yeast histones in the absence (lanes 1 and 2) and presence of Sin3/Rpd3 HDAC complexes purified through Rpd3-TAP (lanes 3 and 4) or Sin3-TAP (lanes 6 and 7), separated by SDS-PAGE, blotted to nitrocellulose, and probed for the presence of H4 phospho-serine 1. While some basal levels of phospho-serine 1 were observed on endogenous yeast histone H4 (lane 2), this signal increased in the presence of Rpd3-TAP (lane 4) or Sin3-TAP (lane 7). (D) Purified CK2 phosphorylates histone H4 Ser1 in vitro. CK2 was affinity purified through Ckb2-TAP and was subjected to kinase assays with wild-type or mutant (S1A) purified yeast histones. As for Sin3/Rpd3, CK2 phosphorylates histone H3 and H4 in vitro, and the ^{32}P-labeled H4 signal is lost on the S1A mutant (top, lane 1 versus lane 2). Western analysis also showed the strong H4 Ser1 kinase activity of CK2 (bottom).

damage, including MMS treatment (21, 24, 27, 36, 37). In light of this, we decided to test tandem affinity-purified Rpd3 in a histone kinase assay. When wild-type yeast histones were incubated with purified Rpd3 complex in the presence of [γ-^{32}P]ATP, histones H3 and H4 were phosphorylated (Fig. 5B, lane 2). Recombinant histone H4 was also efficiently phosphorylated by the Rpd3 complex (lane 3). However, the phosphorylation of H4 was specifically lost when serine 1-to-alanine histone mutant substrates were used (lane 1). To verify whether this phosphorylation event occurred on H4 serine 1, the membrane was blotted with anti-P-Ser1. While the antibody did not recognize the mutated histone H4, an increase in histone H4 phospho-serine 1 was observed in the presence of purified Rpd3 and Sin3 complexes (Fig. 5C, compare lanes 4 and 7 to 2). To confirm that the phospho-serine 1 kinase activity present in Sin3/Rpd3 complexes was in fact CK2, we directly affinity purified the kinase using Ckb2 regulatory subunit, and we tested it in the histone kinase assay (Fig. 5D). Again, phosphorylation of histones H3 and H4 was detected and labeling of H4 was specifically lost in the serine 1 mutant. These data indicate that DNA damage-regulated CK2 is an H4 serine 1 kinase and links histone H4 phosphorylation to its deacetylation by the Sin3/Rpd3 complex. This physical association of histone-modifying activities could ensure that H4 is not acetylated by NuA4 once it has been deacetylated by Rpd3 and phosphorylated by CK2.

Phosphorylation of histone H4 Ser1 is linked to transcription. Yeast proteomic studies have found CK2 associated not only with purified Sin3/Rpd3 but even histones themselves, and also various proteins implicated in transcription (Fig. 6A) (20, 22, 29, 47). Importantly, many of these protein complexes are specifically involved in transcription elongation and have been directly linked to chromatin modification and remodeling. To test whether histone H4 phosphorylation is regulated during the transcription process, we performed ChIPs on the *HSP104* gene before and after heat shock (Fig. 6B). Upon activation of the gene, histone H4 serine 1 became heavily phosphorylated at the coding region, and an increase was also seen at the promoter. Importantly, no variation was detected at the control loci (InterV) or coding regions of two other genes (*PMA1* and *TAF2*). When we looked at the levels of histone H4 acetylation upon gene activation by heat shock, we obtained the opposite results, i.e., a large drop in Ac-Lys8 signals at both the promoter and the coding regions, while other loci were not affected (Fig. 6C). Similar results were obtained for H4 Ac-Lys12 (data not shown). While the interpretation of these results could be complicated by possible loss of histones during the transcription activation process, it clearly correlates deacetylation of histone H4 with its phosphorylation at serine 1. A previous report also found that H4 acetylation was lost during the process of *HSP104* gene activation by heat shock while the presence of unacetylated H4 tails was still detected (11).

Histone H4 serine 1 is phosphorylated near sites of DNA damage in vivo. We have shown that nucleosomal H4 P-Ser1 signals are induced in vivo when cells are treated with DNA damage-inducing agents like MMS (Fig. 4A). As previously mentioned, CK2 itself has been directly implicated in the DNA repair process. For example, it facilitates DNA single- and double-strand break repair through phosphorylation of Xrcc1

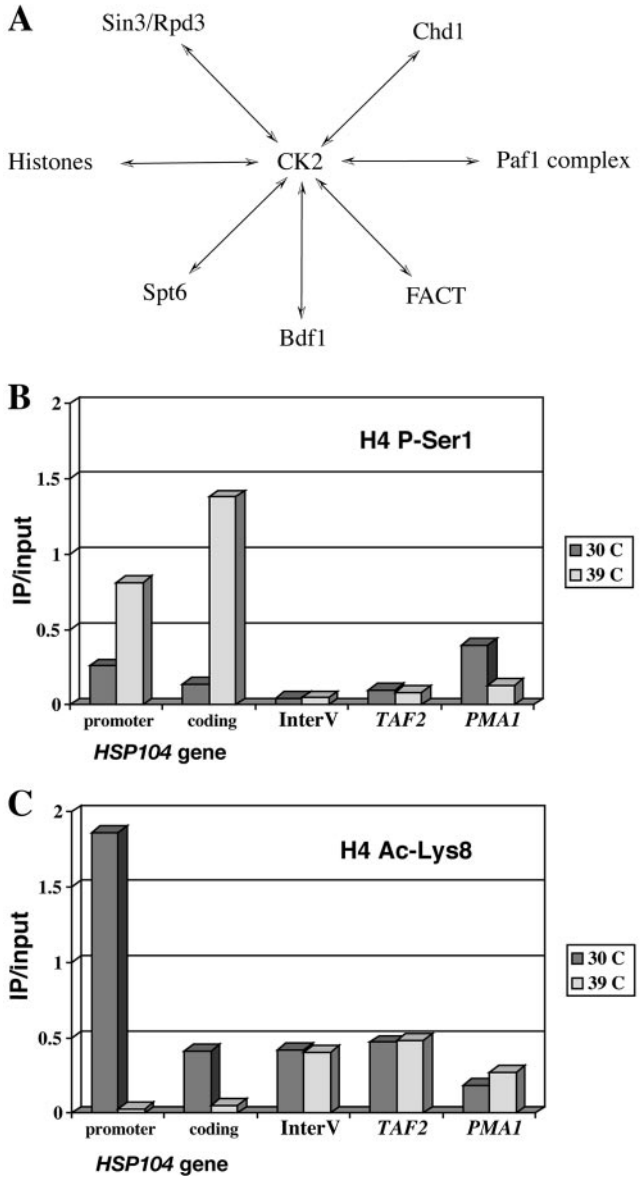

FIG. 6. Phosphorylation of H4 at serine 1 is linked to transcription and correlates with local H4 deacetylation in vivo. (A) Summary of the physical associations detected in proteomic studies between CK2 and transcription regulators. (B) ChIP analysis of H4 P-Ser1 on the *HSP104* gene before and after heat shock. Cross-linked chromatin was prepared from cells grown in normal conditions or incubated for 10 min at 39°C. Immunoprecipitation was performed with anti-P-Ser1 and analyzed by real-time PCR (in triplicate). Ratios of IP to input signal (percent) are presented after subtraction of background signal (no antibody). Results from a control locus and two other genes are also presented (large intergenic region on chromosome V and coding regions of *TAF2* and *PMA1*). Phosphorylation of H4 Ser1 specifically increases over the *HSP104* gene during activation ("promoter" corresponds to the upstream activation sequence, while "coding" is 1.9 kb downstream from the transcription start site). (C) ChIP analysis of H4 acetylation at lysine 8 in the same conditions. In contrast to P-Ser1 in panel B, Ac-Lys8 signals are drastically decreased over the *HSP104* gene upon transcription activation, while the control locus and other genes are not affected.

and Xrcc4 proteins, respectively (27, 36, 37). We were interested to know if phosphorylation of histone H4 by CK2 occurs directly on the chromatin surrounding a DNA damage site in vivo, regulating local NuA4-dependent acetylation. We used a cell system in which expression of the HO endonuclease is under the control of the *GAL* promoter (32). When cells are put in galactose-containing media, HO is expressed and produces a single DNA double-strand break at the *MAT* locus. Since NuA4 has been implicated in DNA double-strand break repair by nonhomologous end joining but not homologous recombination (4), we used a strain in which the latter is not possible (*HML*/*HMR* homologous loci are deleted). Chromatin immunoprecipitations were performed to determine whether H4 was specifically phosphorylated near the HO break. Data are presented as a change of IP ratio to a control locus after the cells are placed in galactose medium. As a positive control, we used an antibody that recognizes histone H2A phosphorylated at serine 129, a histone modification equivalent to mammalian γ-H2AX, known to be important for DNA repair and to specifically appear near a double-strand break in vivo (12, 13, 49). As previously demonstrated, H2A P-Ser129 signal rapidly increases on the chromatin surrounding the DNA break, reaching near-maximum levels within 30 min (Fig. 7A) (12, 49). A strong increase in histone H4 P-Ser1 signal was also measured in the vicinity of the DNA double-strand break (Fig. 7B). On the other hand, phosphorylation of H4 appeared delayed compared to phospho-H2A, as maximum levels were obtained only after 2 to 4 h of HO induction. Interestingly, the appearance of H4 P-Ser1 is concurrent with a decrease in H4 tail acetylation (Fig. 7C). As seen in a separate study analyzing the recruitment of NuA4 (12), H4 is rapidly but transiently acetylated on chromatin surrounding the HO break, in part through NuA4 association with phospho-H2A. Acetylation of H4 lysine 8 increased within 15 min of HO induction, persisted for 60 min, and decreased at later times. These data indicate that in the process of DNA repair, phosphorylation of H4 correlates with its deacetylation, supporting our in vitro results showing inhibition of NuA4 HAT activity by H4 P-Ser1. They also suggest that different histone phosphorylation events regulate NuA4 activity during DNA repair. While phosphorylation of H2A allows NuA4 binding and chromatin acetylation, H4 phosphorylation occurs after NuA4 recruitment and action, at a step of chromatin deacetylation, blocking further action of NuA4. It is tempting to suggest that H4 P-Ser1 plays a role at the step of chromatin restoration after the DNA repair is complete, although in the HO system used here, a large proportion of the DNA breaks are not efficiently repaired (a relatively low level of nonhomologous end joining). Interestingly, similar kinetics for H4 phosphorylation near an HO-induced DNA break has recently been reported in a strain capable of highly efficient repair by homologous recombination (7). Finally, it is important to note that even though H2A is phosphorylated prior to H4 near a DNA break, this event is not a prerequisite for P-Ser1 appearance, as induction of H4 P-Ser1 levels upon MMS treatment is still detected in H2A S129A mutant cells (data not shown).

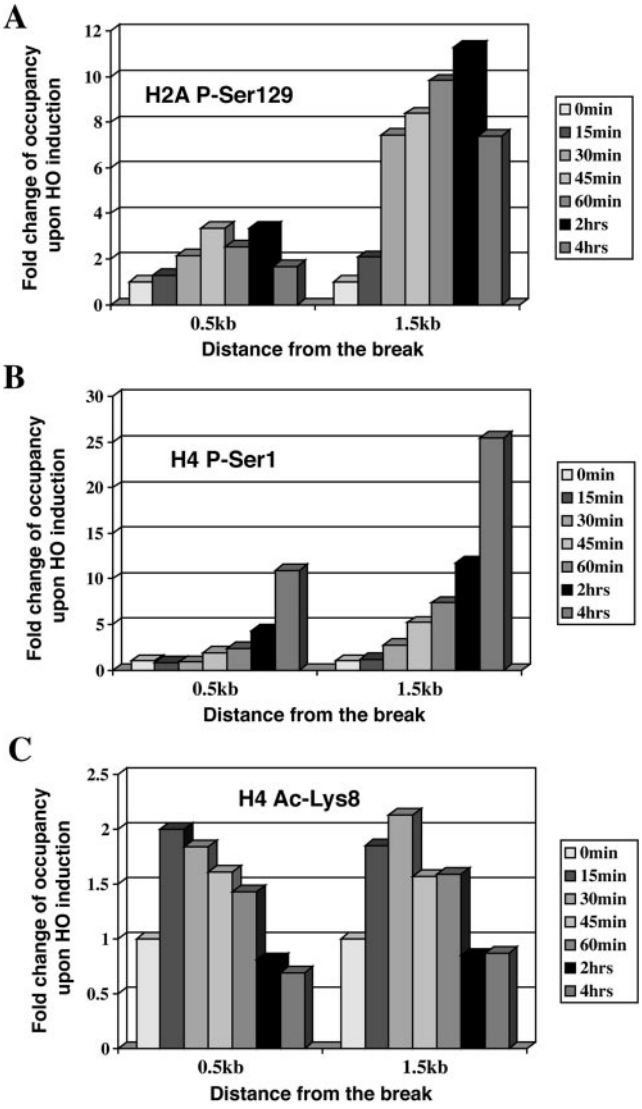

FIG. 7. Nucleosomal histone H4 becomes phosphorylated at serine 1 near a DNA double-strand break in vivo. (A) Cross-linked chromatin was prepared from cells expressing the HO endonuclease under the control of the *GAL* promoter and incubated for the indicated length of time in galactose-containing medium. IPs were performed with anti-H2A P-Ser129 and analyzed by real-time PCR with primers for regions next to the single HO-induced double-strand break at the *MAT* locus. The numbers represent the change of IP ratio to the control locus (InterV) at the different time points compared to time zero (set to 1). In these conditions, the HO cutting site is cleaved more than 80% (data not shown). H2A S129 is phosphorylated to near-maximum levels near the HO break within 30 min. (B) ChIP analysis of H4 P-Ser1 in the same conditions. Histone H4 also becomes phosphorylated near a DNA double-strand break in vivo, but at later time points than H2A. (C) ChIP analysis of H4 Ac-Lys8 in the same conditions. Histone H4 becomes more acetylated in local chromatin upon double-strand break formation but drops at later time points, concomitant with the increase of P-Ser1.

DISCUSSION

Gaining insight into the mechanisms of communication between regulatory proteins/enzymes that modify or interact with the highly conserved histones is crucial to understanding all

levels of DNA-related processes in the cell. It is becoming evident that the local environment of histone modifications is an important factor in dictating which interactions can and will occur. Inherent in this idea are the effects that the various modifications have on structure. Whether it is the general effect of loss of positive charge by acetylation causing loosening of the DNA or a specific local effect, such as phosphorylation of serine 10 on histone H3 affecting or being affected by lysine acetylation/methylation on the same tail, interrelatedness is a key. In addition, certain protein modules, such as bromodomains and chromodomains, have been shown to interact with acetylated lysines and methylated lysines, respectively, in a specific manner.

It was shown that serine 10 phosphorylation on histone H3 is associated with increased acetylation at lysine 14 and transcription activation of a subset of genes (6, 34, 35). In contrast, for the histone H4 tail, our studies demonstrated that phosphorylation of serine 1 has a negative effect on acetylation by NuA4 in vitro. Interestingly, methyl-arginine 3 abrogates this phospho-serine 1 inhibition, though by itself this methylation has little effect on NuA4 acetylation in our peptide assays. On the other hand, methylation of the arginine 3 of histone H4 was previously shown to increase acetylation by human CBP in vitro (independently of other modifications) (53). While PRMT1 was shown to be the enzyme responsible for methylating arginine 3 of histone H4 in mammalian cells, deletion of the yeast homolog Hmt1/Rmt1 does not cause loss of methylation on this residue (30). This suggests that either another protein is responsible for Arg3 methylation in yeast or Hmt1/Rmt1 shares redundant functions with another methyltransferase. Nevertheless, several studies firmly linked mammalian PRMT1 to gene activation in the chromatin context (2, 28, 43, 53). In addition, yeast Hmt1/Rmt1 is recruited to specific genes in vivo during the beginning of the transcription elongation process (55). Recently, an enzyme able to regulate H4 Me-Arg3 levels in vivo by deimination was identified and linked to transcription downregulation (10, 54).

Another point of note is that mammalian histone H2A has the same first five amino acids as H4 and can undergo the same modifications on serine 1, arginine 3, and lysine 5 (3, 30, 46, 50). The protein sequence of both the H2A.Z variant and the *Tetrahymena* H4 tail has an alanine substituted for the usual serine at position 1. This substitution is accompanied by a loss of arginine 3, suggesting that in the absence of serine 1, there is no requirement for arginine 3 (16, 50). Taking this into account with our peptide data, it will be interesting to see whether the role of arginine 3 methylation is somehow related to phosphorylation of serine 1. A critical way to elucidate this relationship will be to obtain antibodies raised against the doubly modified H4 peptide (P-Ser1/diMe-Arg3).

In our analysis of native chromatin, we observed that several nuclear histone modifications, including phosphorylation, acetylation, and methylation of histone H4, are regulated throughout the cell cycle or upon exposure to drugs. After treatment with MMS, which introduces single- and double-strand breaks in DNA, phospho-serine 1 increases while acetylation of histone H4 decreases at lysines 5, 8, and 12 compared to control chromatin. This supports our theory that phospho-serine 1 is inhibitory to acetylation on the histone H4 tail. However, in the case of hydroxyurea-treated cells (blocked in S phase), isolated chromatin was found to have higher levels of phospho-serine 1 with no accompanying decrease in histone H4 acetylation. This discrepancy could be explained as follows. One possibility is that the remaining acetylation exists on different H4 tails than those with the phosphorylated serine. However, considering that modification of neighboring residues can greatly affect the function of an antibody by altering the epitope (Fig. 4B), as is the case for phospho-serine 1 and methyl-arginine 3, it is conceivable that methyl Arg3 is present but not detectable because of serine 1 phosphorylation on the same tail. Additional evidence that serine 1 phosphate is inhibitory to acetylation comes from a study employing mass spectrometry analysis of bulk histones from human cells (19). In that report, the authors never detect phosphorylation and acetylation on the same histone H4 molecule. In fact, upon treatment with okadaic acid, a deacetylation event occurs before phosphorylation is observed, suggesting that the H4 tail must be deacetylated before the phosphate group can be added (19). These data are clearly supported by our finding of an H4 Ser1 kinase associated with the Sin3/Rpd3 complex, the major histone deacetylase in yeast (Fig. 5). Altogether, these results suggest that Sin3/Rpd3 complexes first deacetylate nucleosomal histone H4 tails, followed by Ser1 phosphorylation through associated CK2 activity. This phosphorylation event would ensure that any NuA4 or globally acting picNuA4 complexes (5) could not reacetylate the H4 tail, establishing a more stable deacetylated state of the local chromatin. Accordingly, H4 and H2A P-Ser1 were recently analyzed during early murine development and described as stable "epigenetic" marks in contrast to the more dynamic and reversible acetylation and methylation at arginine 3 (46). Another recent report detected increased H4 and H2A Ser1 phosphorylation in higher eukaryotes during S phase (3), agreeing with the results we obtained in yeast chromatin (Fig. 4) and early work linking P-Ser1 to newly synthesized histone H4 (45). Cheung et al. also independently identified CK2 as a DNA damage-regulated kinase of histone H4 serine 1 (7). This work clearly demonstrates that MMS-induced H4 P-Ser1 signal is lost in *cka1*Δ *cka2*(Ts) double-mutant cells (CK2 has two kinase subunits in yeast, Cka1 and Cka2, and single mutants do not affect H4 P-Ser1 signals) (data not shown and reference 7). Interestingly CK2 was also reported to phosphorylate human histone deacetylases 2 and 3, a modification that promotes both enzymatic activities, again linking the kinase to histone H4 deacetylation (51, 56).

Histone H4 serine 1 and arginine 3 mutants display no obvious growth phenotype and no sensitivity to the DNA-damaging agent MMS (Fig. 5), indicating that these modifications are not by themselves required for cell survival or DNA damage response. Similar results were obtained in a recently published report (7). Interestingly, data presented in this work suggests that phosphorylation of H4 serine 1 slightly affects the efficiency of DNA double-strand break repair by nonhomologous end joining. In any case, the overall weakness or absence of the phenotype of H4 serine 1/arginine 3 mutants suggests that these marks are redundant with other histone modifications, e.g., on the H2A tail. Nevertheless, we clearly demonstrate that H4 P-Ser1 is locally regulated during transcription and DNA double-strand break repair in vivo and its increase correlates with histone H4 deacetylation (Fig. 6 and 7). Im-

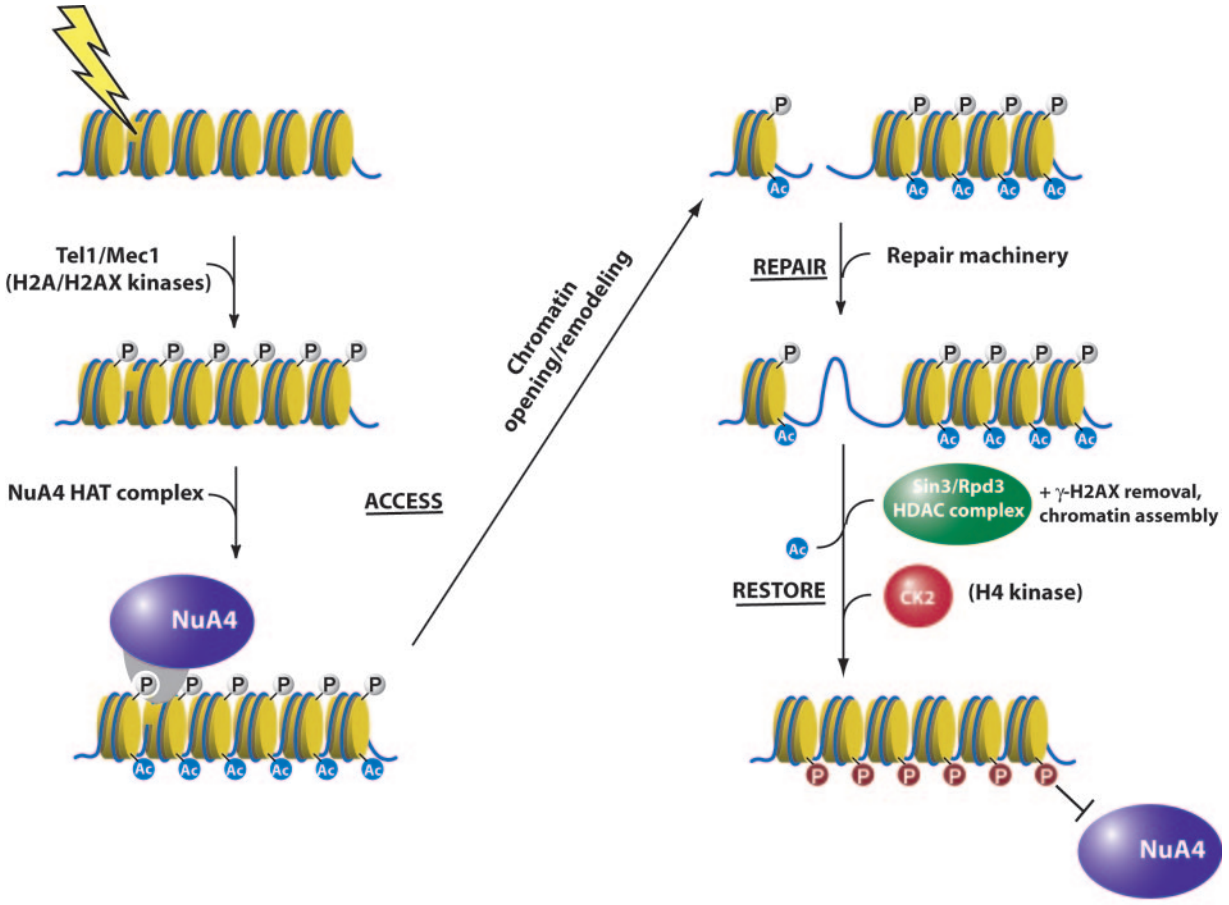

FIG. 8. Model for the interplay of histone phosphorylation events and the NuA4 HAT complex during the repair of DNA double-strand breaks. Appearance of a double-strand break is depicted in the context of chromatin. The proposed access, repair, and restore steps of chromatin modification during DNA repair are indicated. Phosphorylation of H2A(X), recruitment of NuA4, and chromatin acetylation are early events, while chromatin deacetylation and H4 phosphorylation are proposed to occur at the late stage of chromatin reassembly after DNA damage is repaired. In this situation, phosphorylation of H4 blocks local reacetylation by NuA4.

portantly, it has been shown that large transcribed regions of many human and yeast genes were maintained at low levels of histone acetylation compared to the 5′/start site/promoter regions, even though these regions are read by elongating polymerases (33, 42, 44). This accords with the fact that CK2 is found associated with many factors involved in transcription elongation (Fig. 6A). Our ChIP analysis of the *HSP104* gene during activation also correlates histone H4 phosphorylation with deacetylation and transcription. Thus, we hypothesize that the role of H4 phosphorylation/deacetylation during transcription elongation may be linked to nucleosome stabilization after the passage of RNA polymerase II.

Maintenance of genome integrity is another critical nuclear process. The fact that H4 P-Ser1 is directly involved at sites of DNA double-strand breaks in addition to its role in gene transcription indicates that this specific chromatin modification is important in diverse nuclear functions. In a separate study, we previously showed that NuA4 binds to histone H2A phosphorylated on Ser129 near a double-strand break, allowing acetylation of the surrounding chromatin (12). We have now found that histone H4 is phosphorylated at a later stage, after DNA break formation and induction of the damage response (Fig. 7). P-Ser1 appearance correlates again with a decrease of H4 acetylation, and it is reasonable to think that it could be linked to chromatin restoration after DNA repair is complete (41) (a suggested model is shown in Fig. 8). Accordingly, a recent report showed that the Sin3/Rpd3 HDAC complex facilitates double-strand break repair and that histone H4 is deacetylated 4 h after the induction of the HO break (23). The local recruitment of the Sin3/Rpd3 complex (presumably with CK2) between 2 and 4 h postbreak could explain our finding of increased H4 P-Ser1 and a drop in H4 acetylation during that period (Fig. 7). Altogether, these findings indicate that NuA4 function/activity is differentially regulated by two distinct phospho-histone marks (H2A P-Ser129 and H4 P-Ser1) in a stepwise fashion during the process of DNA double-strand break repair (Fig. 8).

In our previous work on the NuA4 HAT complex, we were surprised to find no major variation in its abundance and specific activity during the cell cycle and under other growth conditions (N. Lacoste and J. Côté, unpublished data). In this report, we demonstrate a new efficient way to regulate NuA4 acetyltransferase activity, i.e., through other posttranslational covalent modifications of its substrate, the histone H4 N-ter-

minal domain. These findings add an important new circuitry to the cross talk that occurs between different histone modifications in chromatin and their diverse functional consequences. It also shows how NuA4-dependent acetylation is tightly regulated in vivo, at sites of both gene transcription and DNA repair.

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