Mutational Analysis of H3 and H4 N Termini Reveals Distinct Roles in Nuclear Import*

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Core histones H3 and H4 are rapidly imported into the nucleus by members of the karyopherin (Kap)/importin family. We showed that H3 and H4 interact with Kap123p, histone acetyltransferase-B complex (HAT-B), and Asf1p in cytosol. In vivo analysis indicated that Kap123p is required for H3-mediated import, whereas H4 utilizes multiple Kaps including Kap123p. The evolutionary conservation of H3 and H4 cytoplasmic acetylation led us to analyze the role of acetylation in nuclear transport. We determined that lysine 14 is critical for H3 NLS function in vivo and demonstrated that mutation of H3 lysine 14 to the acetylation-mimic glutamine decreased association with Kap123p in vitro. Several lysines in the H4 NLS are important for its function. We showed that mutation of key lysines to glutamine resulted in a greater import defect than mutation to arginine, suggesting that positive charge promotes NLS function. Lastly we determined that six of ten N-terminal acetylation sites in H3 and H4 can be mutated to arginine, indicating that deposition acetylation is not absolutely necessary in vivo. However, the growth defect of these mutants suggests that acetylation does play an important role in import. These findings suggest a model where cytosolic histones bind import karyopherins prior to acetylation. Other factors are recruited to this complex such as HAT-B and Asf1p; these factors in turn promote acetylation. Acetylation may be important for modulating the interaction with transport factors and may play a role in the release of histones from karyopherins in the nucleus.

Eukaryotic chromatin consists of ~146 base pairs of DNA wrapped around a nucleosomal core composed primarily of the four core histones, H3, H4, H2A, and H2B (1–3). Two copies of each core histone are assembled onto DNA to form a histone octamer. Each core histone has an unstructured N terminus that extends out from the nucleosome and is the target of numerous enzymes, which post-translationally modify specific residues located within the N-terminal tail, altering histone and nucleosomal function (2, 4). Histone acetylation has been well studied and regulates such processes as gene transcription, DNA replication, and DNA repair (4–6). A longstanding mystery has been the role of what has been called deposition acetylation on histones H3 and H4. Histone H4 Lys5,12 diacetylation occurs soon after histone translation and is conserved through evolution (7). Saccharomyces cerevisiae histone H3 is acetylated primarily on Lys9 in the cytoplasm, but this pattern of modification is less conserved (7–9). Recent data suggest that the histone chaperone Asf1p is necessary for acetylation of H3 Lys9 and Lys5, which may occur prior to histone incorporation into nucleosomes (9, 10). Some acetyl groups are removed soon after nucleosome assembly occurs in the nucleus, suggesting a role of early acetylation in either the nuclear import of histones or in nucleosome assembly (11, 12). The H4 Lys5,12 diacetylation pattern is not strictly required for H4 assembly into chromatin and H4 Lys5, Lys9, and Lys12 acetylation appear to play redundant roles (13). The HAT-B (histone acetyl transferase-B)2 complex, found in both the nucleus and cytoplasm, associates with cytoplasmic H4 and is responsible for H4 acetylation (14–16). Gcn5p and Rtt109p have been identified as HATs for unincorporated H3 (9, 17, 18).

Nuclear import of the core histones is important for the efficient progression through the S-phase of the cell cycle, when a ready supply of newly synthesized histones is needed for the assembly of the newly replicated DNA. New histones are likely also required for the chromatin remodeling that takes place during transcription and DNA repair in other phases of the cell cycle. Nuclear import of the core histones is facilitated by a family of proteins known as karyopherins (Kaps) or importins, which are able to bind to nuclear localization signals (NLS) located within nuclear cargo proteins and target the protein for import into the nucleus (19–21). Each core histone contains an NLS in the positively charged N-terminal domain, and indeed many NLSs from diverse proteins have been shown to contain positively charged residues (19–21). As discussed above, the N-terminal domains of histones are the substrates for many modifications that can alter the overall charge status of the N-terminal tail. In budding yeast, the core histones are imported by distinct pathways, with histones H2A and H2B being imported primarily by Kap114p and histones H3 and H4 being imported primarily by Kap123p and Kap121p (16, 22).

We set out to determine which proteins interact with histones H3 and H4 prior to nucleosome assembly, to analyze which amino acids in the histone N terminus were important for import and to determine whether charged or noncharged

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2 The abbreviations used are: HAT, histone acetyltransferase; NLS, nuclear localization signal; GST, glutathione S-transferase; MBP, maltose-binding protein; GFP, green fluorescent protein; wt, wild type; Kap, karyopherin.
NLSs made better import signals. Here we show that the histone chaperone Asf1p and the HAT-B complex proteins (Hat1p, Hat2p, and Hif1p) interact with histones prior to nucleosome assembly. We demonstrate that HAT-B and Asf1p form co-complexes with histones and the transport factor Kap123p, indicating a role for these factors in histone import. We demonstrate that specific lysines in the H3 and H4 N termini, which are known to be acetylated, play critical roles in nuclear import. Using mutants we show that loss of positive charge, by mutation of lysines to glutamine, decreases nuclear import of both H3 and H4 NLS reporters, and in the case of H3 Lys14 decreases Kap123p association. This suggests that acetylation may occur after transport factor binding, or may modulate the interaction of the N-terminal tail with transport factors by preventing transport factors from binding to specific tails in the tetramer.

EXPERIMENTAL PROCEDURES

Yeasts—S. cerevisiae strains used in this study were derived from DF5 and W303 (22). Yeast were manipulated using standard methods described previously (23). JHY200: MATa leu2–3,112 trp1–1 can1–100 ura3-1 ade2-1 his3-11,15 hta1-HTB1::Nat hta2-htb2::HPH hht1-hhf1::KAN hht2-hhf2::KAN pH35 [URA3 CEN ARS HTA1-HTB1 HHT2-HHF2] a generous gift from J.-Y. Hsu and M. M. Smith was previously described (24). JHY200 kap123::HIS3 was created by genomic integration of HIS3 into the KAP123 open reading frame and verified by PCR analysis.

Plasmids—H3 NLS (residues 1–28) and H4 NLS (residues 1–34) constructs were based on the pGFP2-C-FUS plasmid described previously (16). pQQ18 was used to introduce histone mutants into strain JHY200 (24). pQQ18 contains the HTA1-HTB1 and HHT2-HHF2 gene cassettes. N-terminal deletions and specific amino acid substitutions were created by oligonucleotide site-directed mutagenesis. Combination of H3 and H4 mutations was achieved by subcloning the relevant fragments. pMAL-Kap123 and pGEX-H31–28CZ were previously described (16). pGEX-4T1-Hat1 was created by insertion of a DNA fragment containing the HAT1 ORF into pGEX-4T1. pGEX-4T1-Asf1-N was created similarly by inserting residues 1–155 into pGEX-4T1. Histone mutations and plasmid constructs were verified by restriction digest and DNA sequencing.

Microscopy and Quantitation—DF5 yeast transformed with the pGFP2-C-FUS plasmids were grown in SC-MET-URA media for 2 h to induce GFP expression and microscopy was performed as described (16). For each figure, GFP images were captured using the same exposure settings and manipulated identically using Adobe Photoshop 6.0. Quantitation of the nuclear to cytoplasmic (N/C) ratio of mean fluorescent intensity of the histone NLS GFP reporters were performed as described with the following modifications (22). Images were taken at a 500-ms exposure with coincident Hoechst staining to aid in detection of the cell nucleus. The mean N/C ratio was determined for 80–100 cells of each reporter quantified.

Quantitation of GFP Reporters—Whole cell lysates were prepared from equal amounts of induced yeast cultures. Lysates were Western blotted using an anti-GFP antibody, and an anti-Pgk1 antibody as a loading control. GFP and Pgk1p were quantified using a LI-COR Odyssey Infrared Imaging System.

Histone Mutant Strain Construction and Growth Assays—JHY200 yeast strains transformed with plasmid pQQ18 containing mutant alleles of HHT2 and HHF2 were plated onto SC-URA-LEU double selection media to select for both the wild-type plasmid pH33 and the mutant plasmid pQQ18. Multiple colonies from each transformation were grown sequentially on two selection plates of SC media containing 5-fluoroorotic acid (5-FOA) to select against the URA3 positive plasmid covering plasmid. Yeast were struck onto SC-LEU to ensure the yeast contained the LEU2-positive pQQ18 plasmid. After the 5-FOA shuffle, plasmids were recovered and analyzed by restriction digest to verify the yeast retained the correct histone mutations. Cultures of yeast dependent on mutant histone alleles were equalized to an optical density of 1.10-fold serial dilutions of each strain were spotted onto YPD plates and grown at 30 °C for 2–3 days.

Purification of Recombinant Proteins and in Vitro Binding Assays—GST-tagged Hat1p, Asf1-Np, H31–28CZ, H31–28K14QZZ, and MBP-tagged Kap123p were purified according to the manufacturer’s instructions (GE Healthcare, NEB). H31–28CZ and H31–28K14QZZ were cleaved with thrombin as previously described (16). GST-Hat1p and GST-Asf1-Np binding assays were performed in binding buffer (20 mM Hepes pH 7.5, 150 mM KCl, 1 mM EDTA, 0.5% Tween–20) with 15% glycerol. Glutathione-Sepharose was preblocked in TB with 10% bovine serum albumin prior to use in binding assays. Glutathione-Sepharose was preincubated with 500 nM GST-Hat1p and 6 μg chicken erythrocyte histones, or 1 μM GST-Asf1-Np and 2 μg of purified chicken erythrocyte histones, respectively at 4 °C for 1 h. The Sepharose was then washed with binding buffer containing 2 mM KCl. 1 μM MBP-Kap123p was then added and incubated at 4 °C for 1 h. After washing, bound protein was analyzed by SDS-PAGE. H31–28CZ and H31–28K14QZZ binding assays were performed in TB with 15% glycerol as previously described (16). ~3 μg of H31–28CZ or H31–28K14QZZ was incubated with 4.5, 9.0, and 18.0 μg of MBP-Kap123p in the presence of 20 μl of rabbit IgG Sepharose at 4 °C for 1 h as described (16). Bound H31–28CZ and H31–28K14QZZ was detected by staining with Coomassie Brilliant Blue. Bound MBP-Kap123p was detected with anti MBP antibody. Bands were quantitated using a LI-COR Odyssey Infrared Imaging System with Odyssey 2.0 software. Measurements were corrected to loading of the zz protein.

RESULTS

Asf1p and HAT-B Interact with H3 and H4 in Cytosol—We wanted to analyze which proteins interacted with histones H3 and H4 in cytosol, as this may give clues to the acetylation state of histones and the regulatory role of different proteins in histone transport. We have previously published experiments describing the isolation of H4-PrA from cytosolic extracts (16). The interacting proteins that were identified included all four core histones, Kap123p, Pse1p, Hat1p, and Hat2p (16). We have also determined that Hif1p and Asf1p were present in this cytosolic data set (Hif1p; 14 unique peptides, Asf1; 8 unique pep-
Histone NLSs Are Redundant—Both H3 and H4 contain an NLS in their N terminus (16). We therefore wanted to determine whether both NLS signals were necessary in vivo and determine which amino acids played critical roles. It has previously been shown that strains that are dependent for growth on a single copy of H3 and H4 only require one of these histones to have an intact N terminal. These regions were defined as H3 residues 1–28 and H4 residues 2–26 (32), and H3 residues 4–30 and H4 residues 4–28 (33). Histones are thought to be imported as heterodimers or heterotetramers, suggesting that the NLS signals are redundant as each complex will have two or four N-terminal tails. To test this we used the yeast strain JHY200 which bears deletions of both copies of each of the four core histones. The strain is supported by a plasmid pQQ18 that encodes one of each of the core histones. JHY200 grew similarly to its parental wild-type strain, W303, and will from hereon be referred to as wild type. To introduce mutations to the genes encoding histones H3 and H4 (HHT2 and HHF2, respectively) mutations were created in HHT2 and HHF2 in the context of pQQ18 and introduced into the wild type strain by plasmid shuffle. Loss of the H3 NLS (residues 4–30) had a very minor effect on cell growth compared with wild type (Fig. 2). Loss of the H4 NLS (residues 4–28) resulted in viable yeast; however, this strain had a strong growth defect compared with wild type (Fig. 2). These results demonstrated that neither the H3 nor the H4 NLS domains are essential for viability. They also suggested that either the H4 NLS domain had additional important functions in chromatin, or that the H3 N terminus was not a very efficient NLS. However our previous experiments suggest that H3 NLS has robust import activity in reporter assays (16).

We have previously shown that an H3-NLS-GFP reporter is completely mislocalized in Δkap123 strain, suggesting that Kap123p plays a unique role in H3 import (16). We used the strain developed above to test whether Kap123p was necessary to support growth in strains that were reliant solely on the H3 NLS for H3/H4 import. Deletion of KAPI23 alone resulted in a minor growth defect in the JHY200 strain background (Fig. 2). Deletion of KAPI23 did not affect growth in strains dependent

tides). Kap123p and Pse1p were shown to be the major import Kaps for H3 and H4 (16).

The isolation of HAT-B and Asf1p with H4 from cytosolic extracts suggested that they interact with H3 and H4 prior to their nuclear import. However, these immunoprecipitations did not exclude the possibility that multiple complexes existed in yeast cytosol; separate histone-HAT-B or histone-Asf1p complexes, and a histone-karyopherin complex. To confirm that HAT-B and Asf1p existed in import complexes with the karyopherin, we performed in vitro binding assays to determine if a co-complex of Hat1p, histones, and Kap123p can be formed. GST–Hat1p did not interact directly with MBP–Kap123p suggesting it was not a direct cargo of Kap123p (Fig. 1A). We then tested if a co-complex of Hat1p, Kap123p, H3, and H4 could be formed. GST–Hat1p was preincubated with chicken erythrocyte histones prior to the addition of MBP–Kap123p. MBP–Kap123p was able to form a complex with GST–Hat1p in the presence of histones, demonstrating that histones can bridge an interaction between Hat1p and Kap123p in vitro (Fig. 1A). We also demonstrated that histones bridge an interaction between Hat2p and Kap123p (data not shown).

As above we next performed in vitro binding assays with recombinant GST–Asf1p. For these experiments, we used a GST fusion expressing the conserved core region of Asf1, residues 1–155 (GST–Asf1–Np). This region of Asf1p has been demonstrated to interact directly with histones and can rescue defects seen in Δasf1 strains (25–27). Also, GST–Asf1–Np was expressed more efficiently in vitro than full-length GST–Asf1p. MBP–Kap123p did not associate with GST–Asf1–Np, whereas, incubation of this fusion with core histones resulted in the association of H3 and H4 with GST–Asf1–Np. MBP–Kap123p associated with GST–Asf1–Np in the presence of histones, suggesting that histones are bridging an interaction between Asf1p–Np and Kap123p (Fig. 1B). A similar co-complex could be formed using full length Asf1p (data not shown). These results suggest that cytosolic Kap123p exists in co-complexes with HAT-B and Asf1p, and in these complexes the interaction of Kap123p with the assembly factor or HAT is bridged by histones. HAT-B acetylates newly synthesized H4, and Asf1p interacts with histones containing early acetylation marks (14, 28). Asf1p has been shown to be necessary for the addition of early acetylation marks on H3 Lys9 and H3 Lys18 (9, 10, 18, 29–31). A synthetic lethal interaction between Δasf1 and the N terminus of H4 (residues 1–27) has been observed, suggesting that loss of early acetylation marks is deleterious for yeast growth (9). These published results suggested that the acetylated lysines play a crucial role in H3 and H4 import. However it was possible Asf1p and Hat1p were initially recruited to import complexes containing unacetylated histones, and were important to then bring about subsequent histone acetylation. We therefore decided to investigate the role of acetylated amino acids in H3 and H4 in import.

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on the H4 NLS, suggesting that Kap123p was redundant with other Kaps for H4 import. However, deletion of KAP123 in cells dependent on the H3 NLS for H3-H4 import resulted in virtually no yeast recovered after 3 days of growth on 5-FOA containing plates at 30 °C, and only a small number of colonies were visible after longer incubation (Fig. 2 and data not shown). This suggests that H3 import is mediated by Kap123p alone. We also examined strains lacking kap119 and kap108 or with the kap121-34ts, in combination with either an H3 or H4 tail deletion, but saw no striking growth differences (data not shown). This suggests that in contrast to H3, H4 uses several import pathways into the nucleus.

**Amino Acid Residues within the NLS That Are Subject to Acetylation Are Essential in Vivo**—We wanted to determine whether the specific lysines in the H3 and H4 NLS that can be acetylated were essential for viability. We therefore began by creating mutants in which lysines were altered to a charged non-acetylated residue (arginine) or an uncharged residue (glutamine; acetylation mimic). So that we could examine their individual contribution, we mutated the relevant lysines in the context of the one NLS, with the other NLS deleted. We first focused on the H3 NLS as less is known about this NLS, and mutated lysines 9, 14, and 18 to either glutamine or arginine in the context of a strain dependent on the H3 NLS for import. These lysines can all be acetylated prior to nucleosome incorporation, with Lys9 representing the residue most often acetylated (8). We did not recover viable yeast after plasmid shuffle on 5-FOA plates suggesting that these lysines play an essential function in the absence of an H4 N terminus (Table 1). To determine which amino acids were important we also made the lysines 9, 14, or 18 single mutations, and in each case again recovered no viable yeast (Table 1). This suggests that in the absence of the H4 tail, these amino acids are necessary for viability, raising the possibility that one or more is involved in nuclear import.

We also examined the H4 NLS. It has previously been shown that mutation of lysines 5, 8, 12, and 16 together decreases nuclear import; however in this case the H3 tail was present, and there is no evidence that Lys16 is acetylated prior to nucleosome assembly (34). In contrast to H3, no single acetylated lysine in H4 was found to be essential for H4 function. As has previously been reported, mutation of Lys5 or Lys12 singly resulted in no defect, or a very minor growth defect (Table 1) (13). Simultaneous mutation of H4 Lys5 and Lys12 to glycine or arginine also does not result in a growth defect (13). However, we observed that the mutation of all three lysines simultaneously (Lys5, Lys8, and Lys16) cannot support cell growth in the absence of the H3 N terminus (Table 1) as has been previously reported (13). Thus the presence of a lysine in one of these positions is essential for viability. This may be due to the loss of H4 NLS function, and is also likely to be due to loss of crucial acetylation sites necessary for nucleosomal functions. Taken together, these results show that lysines in both H3 and H4, which are known to be acetylated, are necessary for viability in the absence of the other N terminus. This raises the possibility that one or more of these amino acids play a critical role in NLS function. As mutation to both the constitutively charged (Arg) and acetylation-mimic (Gln) had the same effect, it suggested that reversible acetylation was necessary and does not distinguish between acetylation promoting or abrogating transport factor interactions.

**The Histone H3 NLS Contains Lysines Important for Nuclear Localization**—We wanted to test directly whether lysines 9, 14, and 18 in H3 play an important role in nuclear import. As nuclear import is an essential process, and full-length H3 and H4 dimerize, we examined the role of these lysines in the con-
these mutant reporters mirrored the results we had seen with K23 and K27 can also be acetylated in the H3 NLS (8). However, similarly analyzed K23Q, K23R, K27Q, and K27R mutations, as mutation, evident by some cytoplasmic signal (Fig. 3). We also simulated K18Q resulted in only moderate mislocalization, while K18R mutations had little effect on the GFP reporter localization. The triple mutant, suggesting that this mutation abrogates H3 NLS function (Fig. 3). The lysines in H3, Lys9, Lys14, and Lys18, were mutated to Arg or to Glu were expressed in wild-type yeast cells and visualized by fluorescence microscopy. Coincident Hoechst staining is shown.

**FIGURE 3.** Histone H3 NLS contains acetylated lysines that are import for nuclear import. H3 NLS (amino acids 1–28) GFP2, or mutants where the indicated lysines were mutated to Arg or to Glu were expressed in wild-type yeast cells and visualized by fluorescence microscopy. Coincident Hoechst staining is shown.

The text of H3 NLS-GFP fusions described previously (H31–28GFP2) (16). The lysines in H3, Lys9, Lys14, and Lys18, were mutated to arginine or glutamine in the context of the GFP reporter and expressed in wild-type cells. As expected, analysis of the GFP localization demonstrated that wild-type H31–28GFP2 confers nuclear accumulation of GFP, while GFP alone was distributed throughout the cell (Fig. 3). Mutation of Lys9, Lys14, and Lys18 to Arg resulted in the cytoplasmic mislocalization of the GFP reporter when compared with the wild-type H31–28GFP2 reporter (Fig. 3). Mutation of Lys9, Lys14, and Lys18 to Arg resulted in partial cytoplasmic signal, but leaves the nucleus discernable. This result suggested that these key lysines, which are known to be acetylated, play an important role in nuclear import. Interestingly, loss of positive charge, mimicking acetylation, was more detrimental to NLS function, although neither the Arg nor Glu mutations supported viability in strains dependent on the H3 NLS.

Histone H3 K14Q Mutation Leads to Mislocalization of the H31–28GFP2 Reporter—Mutation of individual H3 lysine residues resulted in growth defects in the absence of the H4 NLS (Table 1). To determine whether lysines 9, 14, and 18 were all important for H3 NLS function we therefore mutated individual lysine residues to arginine or to glutamine in the H3 NLS and analyzed their effect on GFP reporter localization. The K9R, K9Q, and K18R mutations had little effect on the GFP localization, while K18Q resulted in only moderate mislocalization, evident by some cytoplasmic signal (Fig. 3). We also similarly analyzed K23Q, K23R, K27Q, and K27R mutations, as K23 and K27 can also be acetylated in the H3 NLS (8). However, these mutant reporters mirrored the results we had seen with mutation of Lys9 and were not analyzed further (data not shown). Surprisingly, mutation of Lys14 to Glu resulted in complete mislocalization of the GFP reporter to the cytoplasm of the cell, similar to that observed with the K9Q/K14Q/K18Q triple mutant, suggesting that this mutation abrogates H3 NLS function (Fig. 3). In contrast to K14Q, K14R resulted in more moderate mislocalization further suggesting the importance of this residue for NLS function. Analysis of GFP reporter expression by quantitative Western blot suggested that most of the reporters were expressed at equivalent levels, although K9Q/K14Q/K18Q was expressed at a higher level than the wild-type reporter, and H3 K9R/K14R/K18R, K9R, and K18R GFP reporters were expressed at slightly lower levels than wild type (data not shown). However increasing or decreasing the induction times of the reporters to vary expression levels did not change the GFP localization (data not shown). Thus, it suggested that Lys14 in H3 plays an essential role in the H3 NLS, which likely explains why Lys14 is essential for viability in the absence of the H4 NLS. Lys14, and to a lesser extent Lys9, may play a more minor role in facilitating the nuclear import of H3, and the fact they were essential in the absence of the H4 NLS points to the fact that they play important roles inside the nucleus. The K14Q and K18Q mutants have a more severe mislocalization phenotype compared with the K14R and K18R mutants, respectively. Therefore, loss of positive charge at Lys14 or Lys18 in the H3 NLS appears to have a negative affect on nuclear import, this is not surprising considering that most NLSs contain positive residues, but contradicts the hypothesis that cytoplasmic acetylation would promote import. Taken together, these results suggest that H3 Lys14 is a critical determinant in the NLS.

The Histone H4 NLS Contains Multiple Amino Acids Important for Nuclear Localization—The first three lysines in the N terminus of H4; Lys5, Lys8, and Lys12, which are also known to be acetylated, were mutated to arginine or glutamine in the context of the GFP reporter. H41–34GFP2 is predominately localized to the nucleus; however some cytoplasmic signal is visible. We chose to use this reporter, rather than the 1–42 reporter used previously, to completely eliminate the possibility of the reporter dimerizing with endogenous H3 (16). In addition, this reporter is more similar to the 1–28 deletion tested in the *in vivo* experiments (Table 1), although both are predicted to contain the H4 NLS (16). Mutation of H4 Lys5, Lys8, and Lys12 to Glu in H41–34GFP2 results in cytoplasmic mislocalization of the reporter, while the H4 K5R/K8R/K12R mutant is not much more cytoplasmic than wild-type H41–34GFP2 reporter (Fig. 4A). The H4 K5Q/K8Q/K12Q GFP reporter was expressed ~2-fold higher levels than the wild-type H4 GFP reporter, but, as with H3 K9Q/K14Q/K18Q, when reporter localization was analyzed following a brief induction, the GFP signal was clearly mislocalized to the cytoplasm (data not shown). This again suggested that charge plays an important role in histone NLS function. However in this case the result was unexpected as Lys5 and Lys12 are acetylated in the cytoplasm. We therefore investigated whether the mislocalization observed was due to mutation of Lys5 and Lys12. The H4 K5Q/K12Q GFP reporter was more mislocalized than the wild-type reporter but less mislocalized than K5Q/K8Q/K12Q suggesting that mutation of Lys5 contributed to the phenotype (Fig. 4A). We also analyzed individual lysine residues that can be acetylated, and GFP expression indicated that nuclear localization of the H41–34GFP2 reporter is slightly decreased in all the lysine to glutamine single mutants (K5Q, K8Q, K12Q, K16Q, and K20Q) (Fig. 4A). We calculated the mean nuclear to cytoplasmic (N/C) ratio of GFP fluores-
cence intensity for selected mutants to more precisely determine differences in localization of the GFP reporters. Wild-type H41–34GFP2 had an observed N/C ratio of 1.83 compared with 1.11 for the H4 K5Q/K8Q/K12Q mutant reporter, indicating that the mutant reporter had higher GFP fluorescence in the cytoplasm (Fig. 4B). The H4 K5Q, K8Q, and K12Q single mutants had N/C ratios of 1.33, 1.24, and 1.24 respectively (Fig. 4B). The differences between the N/C ratios of the H4 single mutants compared with the H4 triple mutant were found to be statistically significant (Student’s t-test, p values of less than 0.0001). The observation that none of the single mutants display such severe mislocalization as observed with the K5Q/K8Q/K12Q triple mutation suggests that these three lysines are contributing in a cumulative manner to the localization of the GFP reporter. Mutation of these three lysine residues to arginine did not affect localization, and interestingly the H41–34K12R-GFP2 reporter was more nuclear than the wild-type H41–34GFP2 reporter. As again, all arginine mutations were more nuclear than glutamine mutations this supported the hypothesis that positive charge is favorable for NLS function and that acetylation would not promote import.

**FIGURE 4.** Histone H4 NLS contains several amino acids that are important for nuclear import. A, H4 NLS (amino acids 1–34) GFP2 fusion was expressed in wild-type yeast cells and visualized by fluorescent microscopy. Specific Lys in the NLS were mutated to Arg or Glu in this reporter and visualized. Coincident Hoechst staining is shown. B, images of cells expressing wild type and H4 NLS GFP mutants were captured, and the mean fluorescent intensity of a defined pixel area was measured in the nucleus (N) and the cytoplasm (C) and used to calculate the mean N/C ratio.

**FIGURE 5.** Mutation of H3 Lys14 to glutamine decreases its interaction with MBP-Kap123p. A, recombinant H31–32GFP2 and H31–32K14Q-GFP2 were immobilized on IgG-Sepharose and binding of increasing amounts of MBP-Kap123p was tested (lanes 1 and 4, 4.5 μg; lanes 2 and 5, 9.0 μg; lanes 3 and 6, 18.0 μg). Bound protein was analyzed by SDS-PAGE. zz-tagged proteins were detected by Coomassie Brilliant Blue. MBP-Kap123p was detected by Western blot using anti-MBP antibodies. Numbers indicate integrated band intensities for each MBP-Kap123p band in arbitrary units. A representative experiment is shown. B, quantitation of integrated band intensities from duplicate experiments. Average intensities are shown.

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Analysis of H3 and H4 Nuclear Import

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Yeast Unable to Acetylate All Six N-terminal Lysines in H3 and H4 Are Viable—Both the H3 K9R/K14R/K18R mutation and the H4 K5R/K8R/K12R mutation resulted in inviability, in the context of yeast lacking the other NLS, suggesting that acetylation of the one remaining N terminus was essential either for nuclear import, chromatin assembly or nucleosomal functions (Table 1). In the context of the GFP reporters the same mutations in H3 led to an import defect, whereas those in H4 did not. We next wanted to test whether acetylation of these residues was necessary for yeast viability. We therefore simultaneously mutated all six lysines to arginine, thus mimicking the constitutively unacetylated state. Mutation of H3 Lys14, and Lys18 to Arg, in the presence of wild-type H4 NLS grew normally, and a strain expressing H4 K5R/K8R/K12R with a wild-type H3 NLS also grew relatively normally (Fig. 6, rows 1 and 2). We showed for the first time that a strain with only one copy of H3 and H4, expressing H3 K9R/K14R/K18R and H4 K5R/K8R/K12R mutations was viable but had a clear growth defect (Fig. 6, row 3). These yeast are viable even with loss of six of ten potential acetylation sites in the N terminus, suggesting that deposition acetylation is not absolutely necessary for histone import.

We were interested to determine however whether the growth defect we observed could be due to decreased import function mediated by the H3 tail as predicted by our GFP studies. To address whether the effect may be partially due to loss of H3 NLS function, we combined our specific lysine mutations with deletion of KAP123. We predicted that deletion of KAP123 in a strain expressing H4 K5R/K8R/K12R with a wild-type H3 NLS would have the same growth defect as one with both the H3 K9R/K14R/K18R and H4 K5R/K8R/K12R mutations, as in both cases the H3 NLS function was abrogated. As shown in Fig. 6 (compare rows 6 and 3), deletion of KAP123 with H3 K9R/K14R/K18R and H4 K5R/K8R/K12R did not produce a further growth defect. In contrast, in the context of wild-type H3 and the H4 K5R/K8R/K12R, loss of KAP123 exacerbated the growth defect (Fig. 6, compare rows 2 and 5). Our GFP assay had suggested that H3 Lys14 played an important role in nuclear import whereas Lys18 was less important. Interestingly, however, in the absence of the H4 tail both were necessary for viability (Table 1). We used the growth assay of strains with and without KAP123 to highlight the different functions of H3 Lys14 and H3 Lys18. Strains expressing H3 K14R and H4 K5R/K8R/K12R had a growth defect in the presence and absence of KAP123 (Fig. 6, compare rows 8 and 10), whereas strains expressing H3 K18R and H4 K5R/K8R/K12R only displayed a growth defect when KAP123 was deleted suggesting that mutation of Lys18 to Arg did not affect import (Fig. 6, compare rows 12 and 14). Therefore, these experiments highlight the important role of H3 Lys14 in NLS function, and suggest that although acetylation is not necessary for NLS function, the ability of Lys14 to become acetylated may be important for efficient import. In contrast, the acetylation of Lys18 appears to be important for functions unrelated to nuclear import.

**DISCUSSION**

In this report, we have shown that histone H3 and H4 interact with HAT-B and Asf1p in cytosol, and can form import complexes with Kap123p and HAT-B and Asf1p. We have determined that the H3 and H4 NLSs are redundant and that Kap123p is necessary in yeast lacking the H4 NLS suggesting a specific role for Kap123p in H3 import. We have shown that loss of any one of three acetylation sites (Lys9, Lys14, or Lys18) in H3 results in inviability in the absence of the H4 N terminus, whereas only simultaneous mutation of H4 Lys5, Lys8, and Lys12 to arginine or glutamine results in inviability in the absence of the H3 tail. We have also shown in the NLS-GFP reporter assay that mutation of key lysines to glutamine results in a greater import defect than mutation to arginine, and that a recombinant H3 acetylation-mimic K14Q NLS associates with HAT-B and Asf1p in cytosol, and can form import complexes, as in both cases the H3 NLS function was abrogated. Previous work has demonstrated that H4 acetylation at Lys5, Lys8, or...
Lys\textsuperscript{12} is necessary for chromatin assembly to occur \textit{in vivo} in the absence of an H3 N terminus (13). This has led to great interest in the function of these acetylation marks. Our initial hypothesis was that histone acetylation would regulate transport by promoting nuclear import, possibly by targeting the histones for import, or by altering their association with Kap proteins. The data presented here using glutamine and arginine substitution mutations, however, suggest that acetylation appears to impair nuclear import.

The HAT-B complex has been identified as the major histone acetyltransferase responsible for acetylation of non-nucleosomal H4 (14, 36). The HAT-B complex is localized to both the cytoplasm and nucleus, and has been linked to chromatin assembly (37, 38). Asf1p is necessary for the efficient acetylation of Lys\textsuperscript{9} and Lys\textsuperscript{36} in H3, however, its site of action is presumed to be nuclear (9, 10, 18, 29–31). Using immunoprecipitation, we have shown that both HAT-B and Asf1p are recruited into a Kap123p-containing histone co-complex from cytosol (16). \textit{In vitro} both Asf1-Np and Hat1p could be recruited to Kap123p in the presence of histones, suggesting that these proteins may be imported with histones. So far we have no evidence that they all form a single co-complex in the cytoplasm however this cannot be ruled out. This complex may be assembled prior to histone acetylation, and then the presence of HAT-B or Asf1p in the complex may directly or indirectly promote histone acetylation. In addition, these proteins would be imported into the nucleus with histones and be available for further nuclear activity during chromatin assembly. So far we have not identified specific import factors for Asf1p and HAT-B, and we have not observed cytoplasmic mislocalization in mutants deleted for single import Kaps.\textsuperscript{3} This suggests that import is dependent on co-import with histones or on several Kaps, or both.

We were also able to show \textit{in vivo} that the H3 and H4 NLS were redundant, consistent with the fact that H3 and H4 are imported as heterodimers or heterotetramers and that both possess an NLS. Surprisingly, loss of the H4 NLS led to a greater growth defect pointing to a specialized function for this domain. As both NLSs work efficiently in reporter assays, we expect this specialized function to be nuclear. We were surprised to determine that yeast lacking the H4 N terminus and dependent on the H3 NLS for import also require Kap123p for growth. This suggested that Kap123p was the only import karyopherin for H3, whereas H4 could be imported by several karyopherins. This result correlated with our previous observation that H3-NLS-GFP was completely mislocalized in the Δkap123 strain (16). We also cannot also rule out the possibility that Kap123p is necessary for the import of an additional factor that is necessary in the absence of the H4 tail.

The H3 N-terminal tails contain several acetylated lysine residues. We showed that in the context of yeast lacking the H4 N terminus, H3 lysines 9, 14, and 18 were necessary for viability suggesting an important function in either nuclear import or in the nucleosome. Interestingly we have shown for the first time the critical role played by Lys\textsuperscript{14} in the H3 N-terminal tail. This amino acid is essential in the context of yeast supported by one tail, and both the K14R and K14Q mutant H3-NLS GFP reporters are mislocalized even though there exist other sites of acetylation in this tail. Our analysis of the H4 NLS, coupled with previous analysis, did not indicate that any specific amino acid, or even Lys\textsuperscript{9} and Lys\textsuperscript{12} together, played a critical role in H4 nucleosomal or nuclear import function (5, 39).

It has previously been reported that when H4 Lys\textsuperscript{5}, Lys\textsuperscript{8}, Lys\textsuperscript{12}, and Lys\textsuperscript{16} were simultaneously mutated to alanine in the context of an NLS GFP reporter an overall reduction of H4 NLS activity was observed (34). This would lead to a great decrease in the overall positive charge in this domain and is consistent with our observations. In agreement with our data, retention of NLS activity was observed with lysine to arginine mutants. The investigators also proposed a role for Cac1p, and to a lesser extent Kap123p in the regulation of nuclear import of their H4 alanine mutant. The authors speculate that Cac1p may be sequestering mutant H4 in the cytoplasm, thus preventing its nuclear import. Our data offer limited support of such a model. We did detect Cac1p interactions with H3 and H4 in the soluble fraction of whole cell lysates (data not shown), but Cac1p was absent from our H4 cytosolic interaction dataset. It may be that Cac1p-H4 interactions are transient and difficult to detect, or the Cac1p-H4 interaction could be occurring within the soluble nuclear fraction.

We observed a marked difference in the NLS-GFP reporter assay between mutating lysines to glutamine or to arginine, and in every case mutation to arginine resulted in more efficient import of the reporter than glutamine. This result suggests that acetylation may impair nuclear import, most likely by decreasing the histone - karyopherin interaction as suggested by our \textit{in vitro} binding data. In the \textit{in vivo} assay even with mutation of all six acetylation sites in the NLSs of H3 and H4 to arginine, thus blocking acetylation, the yeast were viable, albeit with a clear growth defect. The observed growth defect could have been due to nucleosomal defects or to deficient import. However, the fact that deletion of \textit{KAPI23} in the wt H3 / H4 K5R/K8R/K12R mutant background gave the same defect as H3 K9R/K14R/ K18R / H4 K5R/K8R/K12R mutant suggests that an import defect at least contributes to this growth phenotype. As the H3 NLS is presumed to be inactive in these strains, via either Lys\textsuperscript{14} mutation and/or \textit{KAPI23} deletion, these results also suggest that mutation of H4 lysines, 5, 8, and 12 to Arg reduces the NLS activity of the H4 tail. This points to the fact that although positive charge may promote import, the ability to reverse the charge by acetylation is important. Therefore although acetylation is not strictly necessary \textit{in vivo}, it is likely important for efficient karyopherin release or a nucleosomal function.

A major question in the field is what is the role of deposition acetylation seen prior to the incorporation of histones in the nucleosome, such as acetylation of H4 Lys\textsuperscript{5} and Lys\textsuperscript{12} (7). This acetylation mark is conserved from yeast to humans suggesting it is functionally important. We propose a model whereby histones are synthesized in the cytoplasm and then bound in their unmodified state by cognate karyopherins. Other factors are recruited to these complexes such as HAT-B and Asf1p; these factors in turn promote acetylation. Acetylation may serve to help ultimately release histones from transport factors in the nucleus once they have been delivered to chromatin, or to

\textsuperscript{3} J. S. Blackwell and L. F. Pemberton, unpublished data.
recruit additional factors to histones to aid chromatin assembly. Another possibility is that H3-H4 heterodimers or heterotetramers are partially acetylated in the cytoplasm, whereby not all the available acetylation sites in a given tail are modified, or that not every tail is modified. This we predict would serve to modulate karyopherin binding. It is possible that a partially acetylated tail reduces the karyopherin-NLS affinity so that although binding and import occurs it is easily reversed in the nucleus. Alternatively, acetylation of some tails and not others may regulate the number of Kaps that can bind a given heterotetramer or heterodimer, thereby ensuring that not all NLSs are simultaneously recognized leading to the assembly of a very large import complex. In summary, we have analyzed the role of acetylation and shown that while it is not strictly necessary in vivo, our experiments point to it playing to a more subtle role in the modulation of nuclear import or chromatin assembly.

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