The correct assembly of chromatin is necessary for the maintenance of genomic stability in eukaryotic cells. A critical step in the assembly of new chromatin is the cell cycle-regulated synthesis and nuclear import of core histones. Here we demonstrate that the nuclear import pathway of histones H3 and H4 is mediated by at least two karyopherins/importins, Kap123p and Kap121p. Cytosolic H4 is found associated with Kap123p and H3. Kap121p is also present in the H4-PrA-associated fractions, albeit in lesser amounts than Kap123p, suggesting that this Kap serves as an additional import receptor. We further demonstrate that cytosolic Kap123p is associated with acetylated H3 and H4. H3 and H4 each contain a nuclear localization signal (NLS) in their amino-terminal domains. These amino-terminal domains were found to be essential for the nuclear accumulation of H3 and H4-green fluorescent protein reporters. Each NLS mediated direct binding to Kap123p and Kap121p, and decreased nuclear accumulation of H3 and H4 NLS-green fluorescent protein reporters was observed in specific kap mutant strains. H3 and H4 are the first histones to be assembled onto DNA, and these results show that their import is mediated by at least two import pathways.

Core histones comprise the primary protein component of chromatin (reviewed in Ref. 1). These proteins, consisting of histones H2A, H2B, H3, and H4 come together to form the nucleosome when combined with 146 base pairs of DNA (2, 3). The amino-terminal tails of histones radiate from the nucleosome core, whereas the remaining globular portions of the histones are responsible for creating the histone-histone contacts within the core (2, 3). The positively charged amino-terminal tails contain several known sites for posttranslational modification. These include acetylation, phosphorylation, and methylation (reviewed in Ref. 4). Specific modifications are associated with certain DNA template-mediated processes, such as transcription and DNA repair (4).

The deposition of a tetramer consisting of histones H3 and H4 onto DNA constitutes the first step of nucleosome assembly followed by the addition of a pair of H2A and H2B heterodimers to form the histone octamer (reviewed in Ref. 5). Site-specific evolutionary conserved acetylation of the H3 and H4 amino-terminal tails occurs in the cytoplasm, suggesting a role in either deposition or nuclear transport (reviewed in Ref. 6). The histone acetyltransferase B complex, which in yeast resides partially in the cytoplasm, is responsible for cytoplasmic H4 acetylation (7, 8), whereas the enzyme responsible for cytoplasmic H3 acetylation has not been identified.

The import of histones into the nucleus must take place in a rapid cell cycle-regulated manner to efficiently accommodate newly replicated DNA. Although potentially small enough to diffuse into the nucleus, it is unlikely that diffusion would allow the necessary nuclear accumulation of a large amount of histone in a relatively short span of time. The nuclear import of all proteins is believed to be mediated by a family of receptors called karyopherins (Kaps)1 or importins (reviewed in Refs. 9 and 10). Kaps can associate directly with their import cargoes, although examples where adapter proteins are involved have been demonstrated (9). Kaps can also associate with components of the nuclear pore complex, a large macromolecular structure that spans the nuclear envelope and links the cytoplasm and nucleoplasm (reviewed in Refs. 9 and 11) and with the small GTPase Ran (reviewed in Ref. 12). Transport is believed to occur by multiple transient low-affinity interactions between the Kap and the nuclear pore complex, which ultimately leads to translocation of the Kap-cargo complex through the nuclear pore complex (9, 11). Once in the nucleus, import Kaps bind RanGTP, which results in the dissociation of Kaps from their cargoes and leads to the termination of the import cycle (12, 13).

In budding yeast, 14 Kaps have been identified (10, 14, 15). Ten are import receptors, three are export receptors, and one Kap has been shown to be involved in both pathways (10, 14–16). The specific nuclear localization signals (NLSs) recognized by each Kap within their cognate cargoes are mostly undefined, primarily because only one or two cargoes have been determined for each Kap (9). In addition, some cargoes can be imported by a number of different Kaps, suggesting some overlap between NLSs recognized by distinct Kaps (17–21).

We have recently demonstrated that histones H2A and H2B are imported primarily by Kap114p (21), a Kap previously shown to be responsible for the import of the TATA-binding

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¹ The abbreviations used are: Kaps, karyopherins; NLS, nuclear localization signal; GFP, green fluorescent protein; GFP2, two tandem copies of GFP; GST, glutathione S-transferase; MRP, maltose-binding protein; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MS, mass spectrometry; HPLC, high pressure liquid chromatography; N:C, nuclear to cytoplasmic ratio. This paper is available online at http://www.jbc.org
Nuclear Import of Histones H3 and H4

protein (20, 22). As Kap114p is encoded by a nonessential gene and the nuclear function of histones is clearly essential, other pathways of transport exist for H2A and H2B. At least three other Kap are responsible for H2A/H2B import (21). H3 and H4 are the first histones to be deposited onto DNA during the assembly of the nucleosome, and because of their central importance, we sought to determine which factor(s) mediate the import of H3 and H4. In this report, we demonstrate that the import of H3 and H4 is mediated primarily by Kap123p. Kap121p and potentially two other Kaps also serve as pathways of import for these two histones. Cytosolic H4 was found to be associated with Kap123p, Kap121p, and H3. Although presumed to be imported as a heteromer, both H3 and H4 contained NLSs in their amino-terminal domains, which can bind specifically and directly to Kap123p and Kap121p. These amino-terminal domains were found to be crucial for the nuclear accumulation of H3 and H4. The H3 and H4 NLS-GFP reporters were mislocalized to the cytosol in strains harboring mutations in the Kaps, which we identified biochemically. This suggests that several nuclear import pathways are responsible for the nuclear transport of the core histones.

EXPERIMENTAL PROCEDURES

Yeast Strains—Yeast strains in this study were derived from DF5 and manipulated as described previously (23). The protein A-tagged histone H4 strain (H4-PrA) was constructed by integrative transformation of four and a half IgG binding domains of Staphylococcus aureus PrA just upstream of the stop codon of the HHF1 allele (yeast H4 is encoded from two genes, HHF1 and HHF2, which encode identical proteins). The Kap123-PrA strain has been described previously (18). Kap mutant and deletion strains have been previously described: Δkap119 (Δnmfd5) (24), Δkap108 (Δxsm1) (25), Δkap114 (20), Δkap123 (18), Δkap142 (Δnmtn5) (16), kap121ts (pea1-1 in W303) (26), and srrP1 (DsRed2) (17) with the exception of Δkap120, which was a gift of Susana Chaves and Günter Blobel (The Rockefeller University, New York, NY).

Plasmids—GFP reporter constructs were based on pGFP-C-FUS as described previously (21). H3 and H4 reporter constructs were made by cloning the relevant sequences by PCR into pGFP-C-FUS. The NLS domains of H3 (residues 1–28) and H4 (residues 1–42) were expressed as fusions to GST in the amino terminus of two tandem IgG binding motifs of PrA (ZZ tag) at the COOH terminus in pGEX-ZZ (a gift from Michael Nemergut and Ian Macara, University of Virginia). For overexpression of Kap123p in yeast, the KAP123 open reading frame was cloned into p425-GAL1. For purification of recombinant Kap123p, the KAP123 open reading frame was expressed as a fusion to GST in pGEX-4T1.

Cytoplasm Preparation, Protein Isolation, and Western Blotting—Postnuclear postribosomal cytosol was prepared from one liter of the H4-PrA and Kap123-PrA strains as described previously (28). Kap123-PrA and H4-PrA and their associated proteins were isolated by incubation of the cytosol with rabbit IgG-Sepharose as described previously (28, 29). Bound material was washed extensively in transport buffer (21), and proteins were eluted from the IgG-Sepharose with a MgCl2 step gradient, precipitated in methanol, and analyzed by SDS-PAGE. Bands identified by Coomassie Blue staining were excised and analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF) mass spectrometry (MS) as described previously (30). Western blotting was performed by transferring the proteins onto polyvinylidene difluoride membrane and probing with antibodies as noted. The anti-Kap123p antibody was a kind gift from John Aitchison (Institute for Systems Biology, Seattle, WA) (18). The anti-ac-H3 and anti-ac-H4 (Penta) antibodies were kind gifts from C. David Allis (University of Virginia). The binding of antibody was visualized using horseradish peroxidase-conjugated secondary antibody and ECL (Amerham Biosciences, Inc.).

Nano-HPLC Microelectrospray Ionization Mass Spectrometry Analysis and Data Base Searching—For analysis of the entire interacting fraction, IgG-Sepharose and associated proteins were washed extensively in transport buffer and 50 mM MgCl2. Proteins were then eluted with 1 M MgCl2 and precipitated with methanol. An aliquot of the sample was digested with modified trypsin (Promega) at room temperature for approximately 18 h and then stored frozen at −35 °C. An aliquot of the digested sample was acidified and analyzed by nano-HPLC microelectrospray ionization mass spectrometry. All spectra were recorded on an LCQ ion trap mass spectrometer (Thermo Finnigan). Nano-HPLC columns were constructed briefly from 380×75 μm fused silica and packed with 7-μm C18 beads (YMC ODS-AQ, Waters). The HPLC gradient was 0–60% solvent B in 70 min, 60–100% solvent B in 15 min. Solvents A and B were 0.1 m acetic acid in water and 0.1 m acetic acid in 70% acetonitrile, respectively. Spectra were acquired with the instrument operating in the data-dependent mode throughout the HPLC gradient. Every 12–15 s, the instrument cycled through the acquisition of a full scan mass spectrum, and 5 MS/MS spectra recorded sequentially on the five most abundant ions present in the initial MS spectrum. These spectra were searched against S. cerevisiae gene data base using SEQUEST (31, 32). Peptides with cross correlation scores >2 were manually confirmed. Peptides were synthesized by the solid phase method using standard fmoc chemistry, purified by reverse phase HPLC, and analyzed by mass spectrometry to confirm their purity and sequence.

Cell Culture and Microscopy—Strains containing the reporter constructs based on pGFP-C-FUS were grown and induced as described previously (21). All microscopy and image manipulation were performed as described previously (21) with a Nikon Microphot-SA microscope and OpenLab software using a ×60 (in figures) or ×100 (for quantitation) objective. Quantitation of nuclear to cytoplasmic (N:C) fluorescence intensity ratios were determined as described previously (21). For each figure, images were taken at the same exposure settings to ensure identical fluorescence levels. The ratio of H4 and COOH-terminal GFP reporter constructs (H5–58–136, H8–83–138, H4–42–103, H4–81–103), which were taken at higher exposure times and manipulated separately.

Recombinant Protein Expression and Binding Assays—MBP-tagged Kap123p and GST-tagged Kap121p were purified by overexpression in Escherichia coli and purified on amylose-agarose or glutathione-Sepharose resin, respectively, according to the instructions of the manufacturer (New England Biolabs) for MBP-Kap123p or as previously described for GST-Kap121p (21). The GST tag was cleaved from purified Kap121p with thrombin. The H3 and H4 NLSs were expressed as GST-NLS-ZZ fusions, purified on glutathione-Sepharose, and cleaved with thrombin while bound, releasing the NLS-ZZ fusion protein. For each binding reaction, ~5 μg of NLS-ZZ fusion protein was incubated with 2.5 μg of MBP-Kap123p or 2.5 μg of Kap121p and 20 μl of rabbit IgG-Sepharose at 4 °C for 1 h. The IgG-Sepharose was preblocked with 10% bovine serum albumin in transport buffer. The Sepharose was then washed extensively with transport buffer and boiled in SDS sample buffer, and bound material was analyzed by SDS-PAGE and Coomassie Blue staining.

RESULTS

Histone H4 Interacts with Acetylated H3, Kap123p, Kap121p, and a Histone Acetyltransferase Complex in Yeast Cytosol—To identify the import receptor for histone H4, H4 and its interacting partners were isolated from cytosol. A haploid yeast strain was constructed where one copy of H4 (HHF1) was COOH-terminally tagged with PrA and used to prepare postribosomal cytosol. To isolate H4-PrA and associated proteins, this extract was incubated with IgG-Sepharose, and isolated proteins were eluted with a MgCl2 step gradient and analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 1a). H4-PrA was eluted at the highest MgCl2 concentration (4.5 M) as expected, and a number of distinct bands, presumably H4-associated proteins, were eluted at lower concentrations of MgCl2. The amount of H4-PrA was less than expected and was probably because of insolubility after MgCl2 elution, as with H2A and H2B (21). A band that corresponded to a size expected of a karyophilin (~116 kDa) eluted in the 100–1000 mM MgCl2 fractions. Initial characterization of the excised band found in the 250 mM fraction was carried out by MALDI-TOF mass spectrometry, which revealed that it contained Kap123p, suggesting that H4 and Kap123p specifically interact in cytosol. This result was confirmed by Western blotting a parallel set of H4-PrA-associated fractions (0–1000 mM MgCl2) with an antibody to Kap123p (Fig. 1a). The interaction of

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Kap123p and H4 suggested that Kap123p may be the primary import receptor for H4.

The fact that the Kap123 gene is nonessential suggested that other pathways of import must exist for H4. To determine whether other Kaps besides Kap123p were present in the ~116-kDa bands in the 100–1000 mM MgCl₂ fractions, an analysis of these bands was carried out using liquid chromatography/tandem mass spectrometry. Using this more sensitive technique, numerous peptides for Kap123p were found for the ~116-kDa bands (from elutions with 100 mM, 250 mM, and 1 M MgCl₂), confirming the MALDI-TOF MS data. Additionally, a number of peptides corresponding to Kap121p was found in each of the same bands. This suggested that Kap121p may serve as an additional import pathway for H4. Peptides corresponding to Nmd5p/Kap119p were also found in the 1 M MgCl₂ fraction, suggesting that this Kap may serve as a minor import receptor for H4.

The above MS analysis was limited to the visible gel bands migrating at ~116 kDa in the 100–1000 mM MgCl₂ fractions. However, a number of other Kap proteins have significantly lower or higher molecular weights, which may have been missed by this type of selective analysis. To determine the H4-PrA-interacting proteins more comprehensively, two parallel experiments were carried out using cytosol from this strain. After washing the cytosolic H4-PrA-associated proteins with 50 mM MgCl₂, all associated proteins were eluted with 1 mM MgCl₂ and precipitated. In the first experiment, this fraction was analyzed by SDS-PAGE, and the gel region corresponding to 90–150 kDa (which includes all the yeast β-Kaps) was analyzed by liquid chromatography/tandem MS as described above. In the second experiment, the entire precipitated fraction was analyzed by liquid chromatography/tandem MS. From both experiments, numerous peptides corresponding to Kap123p and Kap121p were found. In addition, a single peptide corresponding to Sxm1p/Kap108p was found in the MS analysis of the 90–150-kDa region. The consistent presence of Kap123p and Kap121p in all of these fractions strongly suggested that these two Kaps constitute the major pathway of import for histone H4. In addition, the isolation of peptides (albeit relatively few) from Nmd5p/Kap119p and Sxm1p/Kap108p suggested that several other Kaps may play a minor role in H4 import.

To determine whether H3 was present in the H4-PrA-associated fractions, a parallel set of H4-PrA-associated fractions was blotted with an antibody that recognizes acetylated H3. This showed that H3 is indeed associated with cytosolic H4 (Fig. 1a) and suggested that H3 and H4 may be co-imported by Kap123p. H3 was also found to be present when analyzing the entire 1 mM MgCl₂ H4-PrA-interacting fraction with liquid chromatography/tandem MS. These two proteins had been previously identified as a cytosolic H4-associated acetyltransferase complex (7). This finding suggested that cytosolic Hat1p/Hat2p existed in a complex with H4, however, it is possible that this complex may be distinct from the H4-Kap123p complex.

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Cytosolic Kap123 Is Associated with Acetylated H3 and H4—To confirm that H3 and H4 are indeed cargoes for Kap123p, cytosol from a Kap123-PrA strain was isolated and incubated with IgG-Sepharose, and associated proteins were eluted with MgCl₂ as described above for the H4-PrA experiments. Initially, fractions were analyzed by SDS-PAGE and stained with Coomassie Blue. However, because of the number of Kap123p-interacting proteins present, it was not possible to identify which bands represented H3 and H4. This was not surprising as Kap123p interacts with and imports at least 20 ribosomal proteins (18). Thus, Kap123-PrA-associated frac-
The amino-terminal domains of H3 and H4 are necessary for the nuclear accumulation of H3 and H4. Full-length or various fragments of H3 (a) or H4 (b) lacking the amino-terminal domain, as indicated by amino acid number, were expressed as fusions to two tandem repeats of GFP (GFP<sub>2</sub>) in wild-type yeast cells and visualized by fluorescence microscopy. The coincident Hoechst staining is shown. The schematic representation of the structures of H3 and H4 are shown drawn to scale (top). T, amino-terminal tail; α, α-helix; αN, amino-terminal α-helix.

H3 and H4 Each Contain a Nuclear Localization Signal at Their NH<sub>2</sub> Termi—We previously demonstrated that H2A and H2B each contain a NLS located at their amino termini, and that these sequences are sufficient for association with the Kaps responsible for their transport (21). Because of the overall similarity in histone structure, we hypothesized that histones H3 and H4 also contained NLSs in their amino termini. To test this hypothesis, amino acids 1–58 of H3 and 1–42 of H4 were expressed as fusions with two tandem copies of GFP (GFP<sub>2</sub>) in yeast. An analysis of the expression of both reporters demonstrated that these residues were sufficient to confer nuclear accumulation of GFP, whereas GFP alone was localized over the entire cell (Fig. 2, and data not shown). Smaller amino-terminal GFP fusions were constructed to determine the minimal NLS for each histone. For H3, the minimal region capable of conferring nuclear localization was residues 1–28 (Fig. 2a). Further deletion from either the NH<sub>2</sub> or COOH terminus (residues 1–20 or residues 13–28) abolished NLS function. For H4, the minimal NLS domain was mapped to residues 1–21 (Fig. 2b). The NLS function of the amino terminus of H4 was abolished when the first 11 residues of this domain were deleted (Fig. 2b). The minimal NLS of H4 (residues 1–21) did not appear entirely nuclear, and more cytoplasmic signal was visible than had been detected with the H4-(1–42) GFP<sub>2</sub> reporter. This finding suggests that residues 22–42 of H4 contain determinants that may contribute to NLS function. Taken together, these results suggest that both H3 and H4, like H2A and H2B, contain functional NLSs at their amino termini.

The Amino-terminal Domains of H3 and H4 Are Necessary for the Nuclear Accumulation of H3 and H4—The experiments described above demonstrated that the amino-terminal domains of H3 and H4 are sufficient for the nuclear accumulation of these histones. To determine whether the amino termini of H3 and H4 are necessary for their nuclear accumulation, portions of these proteins, which lacked these regions, were expressed as fusions to two tandem repeats of GFP as noted above. As expected, the full-length H3 and H4 reporter constructs were exclusively nuclear (Fig. 3). The expression of H3 and H4 reporters that lacked their respective amino termini (H3-(58–136) and H4-(42–103)) resulted in a loss of nuclear accumulation with the reporters staining both the nucleus and cytoplasm. Deletions that removed successive histone α-helices from the amino terminus of these reporter constructs (H3-(83–136), H3-(118–136), and H4-(81–103)) also resulted in the staining of the cytoplasm and nucleus as expected. These data suggested that additional NLS determinants are not present in the COOH-terminal half of histones H3 and H4. Thus, the amino-terminal domains of H3-(1–58) and H4-(1–42) are necessary and sufficient for interaction with the nuclear transport machinery in vivo.

Kap123p and Kap121p Bind Directly to the NLSs of H3 and H4—Our fractionation experiments from yeast cytosol suggested that Kap123p and Kap121p interacted with cytosolic H3 and H4. To determine whether Kap123p and Kap121p interacted directly with the NLSs of H3 and H4, the proteins were expressed in bacteria, and in vitro binding assays were carried out. The NLSs of H3 and H4 (H3 residues 1–28 and H4 residues 1–42) were purified as fusions to GST at the amino terminus...
Nuclear Import of Histones H3 and H4

Fig. 4. Kap123p and Kap121p can associate directly with the amino-terminal domains of H3 and H4. Recombinant MBP-tagged Kap123p or tag-free Kap121p was incubated with ZZ tag alone (zz), H3 residues 1–28 fused to the ZZ tag (H3-zz), or H4 residues 1–42 fused to the ZZ tag (H4-zz) in the presence of IgG-Sepharose. Bound material was analyzed by SDS-PAGE and visualized by Coomassie Blue staining. The input lane represents 25% Kap protein used for each binding reaction.

and a ZZ tag (two tandem repeats of the PrA IgG binding domain) at the COOH terminus. The GST tag was removed, and binding assays were carried out using the NLS-ZZ fusion proteins immobilized on IgG-Sepharose or the ZZ tag alone as a negative control. Recombinant MBP-Kap123p or untagged Kap121p was added to each binding reaction, and bound material was analyzed by SDS-PAGE and Coomassie Blue staining. As shown in Fig. 4, Kap123p was able to bind specifically to the H3 and H4 NLS sequences but not to the tag alone. The relative amount of Kap123p that bound the H3 NLS appeared higher than the amount that bound the H4 NLS. Kap121p was also able to bind both NLSs (Fig. 4). These data supported the notion that Kap123p and Kap121p interact directly with both H3 and H4 via their amino termini and suggested that these Kaps recognize the same or overlapping sequences in H3 and H4. This finding is consistent with the proposed role for these Kaps as the major import factors for these histones.

At Least Two Kaps, Kap123p and Kap121p, Mediate the Import of H3 and H4—To determine the in vivo role of Kap123p, Kap121p, and potentially other Kaps in the import of H3 and H4, NLS-GFP reporters of H3 and H4 were analyzed in cells harboring deletions or mutations in specific Kap genes. In addition, we quantitated the mean N:C ratios of GFP fluorescence intensity for both reporters in the different strains. The nuclear accumulation of the H3-(1–28)GFP2 reporter was clearly decreased in ∆kap123 cells in comparison to its nuclear localization in wild-type cells (Fig. 5a). The mislocalization of this reporter could be reversed by the overexpression of Kap123p in the ∆kap123 cells (data not shown). This same reporter was not significantly mislocalized in another kap mutant, ∆kap114. The quantitation of the N:C ratios of GFP fluorescence in these strains reflected this observation (Fig. 5b). In wild-type and ∆kap114 cells, the mean N:C ratio was >3.6:1, which was similar to the N:C ratio in ∆kap114 cells (~3.6), but the N:C ratio of this reporter in ∆kap123 cells (1.05:1) reflected the equalization of this reporter to both the nuclear and cytoplasmic compartments. We observed no decrease in nuclear accumulation of the H3-(1–28)GFP2 reporter in cells harboring deletions in kap120/lph2, kap108/sxm1, kap142/msn5, or in a strain harboring the kap60 temperature-sensitive allele srp1–31 (data not shown). An analysis of this reporter in a kap121 temperature-sensitive strain (pse1–1) revealed a slight decrease in nuclear accumulation of this reporter relative to wild-type cells (Fig. 5a). A similar degree of decreased nuclear accumulation was observed in the ∆kap119 strain. The quantitation of the N:C ratio of this reporter in both of these strains revealed a decrease relative to wild-type cells (Fig. 5b), although the N:C ratio was still significantly higher in the ∆kap123 strain underscoring the relative importance of this Kap in the import of H3.

Parallel experiments were carried out to determine the role of the same Kaps in the import of H4. The H4-(1–42) GFP2 reporter was used in these experiments as the shorter NLS-GFP reporters gave significantly more cytoplasmic staining in wild-type cells, making subtle effects on localization difficult to observe. In ∆kap123 cells, more cytoplasmic GFP signal was evident with the H4-(1–42) reporter relative to wild-type cells (Fig. 5c), although the degree of mislocalization was not as dramatic as with the H3-(1–28)GFP2 reporter. These observations were reflected in the quantitation of the N:C ratios of GFP fluorescence with this reporter with wild-type cells having a mean N:C ratio of 4.2:1 and ∆kap123 cells having a mean N:C ratio of 3.0:1 (Fig. 5d). A similar increase in the cytoplasmic GFP signal was not observed in the ∆kap114 or ∆kap119 strains, although the mean N:C ratios were slightly lower than in wild-type cells. As with the H3-(1–28)GFP2 reporter, no decrease in nuclear accumulation was observed with the H4 reporter in cells harboring deletions in kap120/lph2, kap108/sxm1, kap142/msn5 or in the srp1–31 ts strain (data not shown). An analysis of this reporter in the kap121 ts strain revealed a small degree of cytoplasmic mislocalization relative to wild-type cells (mean N:C ratio of 3.3:1). These results demonstrated that in vivo Kap123p plays a major role in the import histones H3 and H4 and also suggested that other Kaps, such as Kap121p, may function in this pathway.

DISCUSSION

In this report, we have elucidated the major pathways of nuclear import for histones H3 and H4. Co-immunoprecipitation experiments of cytosolic extracts revealed that H4 is bound to H3, Kap123p, and Kap121p, suggesting that these two Kaps are the major receptors responsible for the import of these two histones. We have also demonstrated that H3 and H4 each contain an NLS at their amino-terminal domains, which can mediate direct interaction with Kap123p and Kap121p. These domains are necessary for the nuclear accumulation of H3 and H4 because reporters, which lack these domains, lost the ability to localize to the nucleus.

We had demonstrated previously that histones H2A and H2B each contain an NLS at their amino-terminal domains (21). Our in vivo localization and in vitro binding studies described here demonstrate that H3 and H4 also contain NLSs in the analogous domains. Therefore, each yeast core histone contains at least one NLS in the amino-terminal domain. Interestingly, this domain of the histone is reversibly modified by several posttranslational modifications (4) and thus may provide a mechanism to regulate Kap association. Further deletion analysis of H3 and H4 demonstrated that the COOH-terminal globular domains of H3 and H4 do not contain NLS activity, and hence, the amino-terminal domains of H3 and H4 are necessary for nuclear accumulation of these histones. A recent study (33) demonstrated that in human cells, NLS activity is found in the amino-terminal domains of all core histones. Histones, particularly H3 and H4, are nearly identical from yeast to humans, suggesting that the amino-terminal NLSs will be very similar. However, contrary to the data presented here, NLS activity was also found in the COOH-terminal globular
domains of the core histones in the same study (33). It is possible that additional Kaps not present in yeast recognize the globular domains of histones in human cells. Alternatively, as the globular domains mediate heterodimerization, it is possible that the observed nuclear accumulation was the result of interaction with endogenous histones, which would mediate nuclear import (33). It may be that this interaction did not occur in the context of our COOH-terminal GFP2 reporter fusions, or that a domain more amino-terminal to this region is needed to mediate heterodimerization. Nevertheless, NLS activity was not detected in the COOH-terminal domains of H3 and H4 in our system.

In our co-immunoprecipitation experiments using H4-PrA cytosolic extract, we also found cytosolic H4 bound to the previously characterized Hat1p-Hat2p acetyltransferase complex (7). It has been shown that Hat1p-Hat2p interacts with H4 via its amino-terminal tail (7). As we have shown that Kap123p as well as Kap121p interact directly with the amino-terminal domain of H4, the Kap-histone complex may be distinct from the Hat1p-Hat2p-histone complex, because they appear to bind to overlapping domains. We have also demonstrated that at least a fraction of the Kap123p-associated H3 and H4 is acetylated, suggesting that an interaction of the histones with the acetyltransferase occurs prior to Kap binding. It is interesting that the NLSs, which we have mapped, contain most of the known cytoplasmic acetylation sites on H3 and H4 (4). Some of these site-specific posttranslational modifications are conserved from human to yeast, and although their exact function is not known, it has been postulated that they may function in import or in assembly of histones onto DNA as chromatin (6).

Future studies will determine whether acetylation is necessary for the interaction with these Kaps perhaps providing a reversible mechanism for the regulation of Kap binding. In addition, although originally described as a cytoplasmic histone acetyltransferase complex, a nuclear pool of the Hat1p-Hat2p complex has also been demonstrated (7, 8). This suggests that these proteins must also be imported into the nucleus, most likely via a Kap-mediated pathway. In vitro binding experiments have so far failed to demonstrate a direct interaction between Hat1p-Hat2p and Kap123p, and it is possible that another Kap mediates import of this complex.2 Co-import of the Hat1p-Hat2p complex by association with H4 remains an alternative possibility.

Although it encodes a nonessential Kap, Kap123p was originally identified as the major nuclear import receptor for ribosomal proteins (17, 18). Interestingly, Kap121p was found to be a secondary import pathway for ribosomal proteins, because it could substitute for Kap123p in the import of a ribosomal protein NLS reporter (17, 18). The presence of Kap121p in association with cytosolic H4 and the relative decrease in the nuclear accumulation of the H3 and H4 NLS-GFP reporters in a kap121 temperature-sensitive strain suggested that this Kap also plays a role in the import of these histones. The fact that significantly more Kap123p than Kap121p was found in association with H4 as well as a more dramatic decrease in the nuclear accumulation of the H3 and H4 NLS-GFP reporters suggests that Kap123p constitutes the major import Kap for both of these proteins. We do not know why the H3 NLS-GFP reporter was more cytoplasmic in the Δkap123 strain, suggesting that the H3-NLS may not be recognized effectively by other import Kaps in vivo. Nevertheless, the greatest decrease in the nuclear accumulation of the H4 NLS-GFP reporter was observed in the Δkap123 strain, suggesting that in vivo this Kap is also primarily responsible for the import of H4. Kap123p is the most abundant karyopherin (18) and may represent a specialized import re-
ceptor for small highly abundant nuclear proteins such as ribosomal proteins and histones (17, 18). The only other Kap123p cargo whose NLS has been analyzed is the ribosomal protein L25 (18, 34). Kap123p may also serve as a minor import Kap for H2A and H2B (21). A comparison of these NLSs to the NLSs of H3 and H4 reveals little significant homology with the only common feature being the relatively high number of positively charged residues in all of these sequences. Relatively, the greatest degree of homology was observed between the amino-terminal domains of H2A and H4. We do not know whether this reflects a greater level of similarity between their NLSs or other shared functions of the H2A and H4 amino-terminal domains. Future studies will determine whether Kap123p has distinct binding sites for histones and ribosomal proteins allowing these cargoes to be imported simultaneously, or whether they compete for binding.

The data we present here combined with our previous studies suggest that the core histones are imported primarily by two Kaps with Kap123p being responsible for the import of H3 and H4, and with Kap114p serving as the primary receptor for the import of H2A and H2B (21). Because both of these Kaps are nonessential, additional pathways of transport must exist for the import of histones. We had previously demonstrated that a number of Kaps serve to import H2A and H2B in the absence of Kap114p (21). The presence of Kap121p, Kap119p, and Kap108p as revealed by mass spectrometry in the H4-PrA-associated fractions suggested that these Kaps may serve as alternative pathways for the import of H4 and perhaps for H3 as well. It is probable that in mammalian cells several Kaps may mediate the import of these two histones. Indeed, a report, which was published while our studies were under review, demonstrated that a number of mammalian Kaps can bind to the core histones and mediate their nuclear import in vitro, although the relative contribution of these Kaps to histone import in vivo was not established (35). Although we do not have evidence to support the hypothesis, it is possible that the import of H3 and H4 could also be mediated indirectly by association with other import cargoes. Alternatively, individual histones are small enough to diffuse through the nuclear pore complex. However, because H3 and H4 form a heterotetramer with a molecular mass of >50 kDa, it is unlikely that diffusion plays a significant role in the transport of H3 and H4. Indeed, the import of histones in vertebrate systems has been shown to be an energy-requiring process (19, 33, 36, 37).

In summary, we have shown that both yeast histones H3 and H4 contain an NLS in their amino-terminal domains, and that import of these histones is primarily mediated by the association of Kap123p with these sequences. Kap121p and perhaps a number of other kars serve as additional receptors for the import of these histones. H3 and H4 are the primary building blocks of the nucleosome, and the determination of the nuclear import pathway of H3 and H4 has elucidated an otherwise unexplored aspect of histone metabolism.

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REFERENCES
1. Kornberg, R. D., and Lorch, Y. (1999) Cell 98, 285–294
2. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997) Nature 389, 251–260
3. White, C. L., Sato, R. K., and Luger, K. (2001) EMBO J. 20, 5207–5218
4. Stryahl, B. D., and Allis, C. D. (2000) Nature 403, 41–45
5. Ito, T., Tyler, J. K., and Kadonaga, J. T. (1997) Genes Cells 2, 593–600
6. Verreault, A. (2000) Genes Dev. 14, 1430–1438
7. Pelham, H. R., Widom, J., and Gotteiching, D. E. (1996) Cell 87, 85–94
8. Ruiz-Garcia, A. B., Sendra, R., Galiana, M., Pamblanco, M., Perez-Ontin, J. E., and Tordera, V. (1998) J. Biol. Chem. 273, 12599–12605
9. Nechkat, S., and Dreyfuss, G. (1999) Cell 96, 677–690
10. Pemberton, L. F., Blobel, G., and Rosenblum, J. S. (1998) Curr. Opin. Cell Biol. 10, 382–399
11. Rout, M. P., and Aitchison, J. D. (2001) J. Biol. Chem. 276, 16593–16596
12. Moore, M. S. (1998) J. Biol. Chem. 273, 22857–22860
13. Rexach, M., and Blobel, G. (1995) Cell 83, 683–692
14. Gorlich, D., Dobrowski, M., Bischoff, F. R., Kutay, U., Bork, P., Hartmann, E., Pfehr, S., and Izaurralde, E. (1997) J. Cell Biol. 138, 65–80
15. Wozniak, R. W., Rout, M. P., and Aitchison, J. D. (1998) Trends Cell Biol. 8, 184–188
16. Yoshihda, K., and Blobel, G. (2001) J. Cell Biol. 152, 729–740
17. Schlenstedt, G., Smirnova, E., Deane, R., Solbach, J., Kutay, U., Gorlich, D., Pongstingl, H., and Bischoff, F. R. (1997) EMBO J. 16, 6237–6249
18. Rout, M. P., Blobel, G., and Aitchison, J. D. (1997) Cell 88, 715–725
19. Jakel, S., and Gorlich, D. (1998) EMBO J. 17, 4491–4502
20. Pemberton, L. F., Rosenblum, J. S., and Blobel, G. (1999) J. Cell Biol. 145, 1407–1417
21. Mosammaparast, N., Jackson, K. R., Guo, Y., Brane, C. J., Shahanowitz, J., Hunt, D. F., and Pemberton, L. F. (2001) J. Cell Biol. 153, 251–262
22. Morehouse, H., Buratowski, R. M., Silver, P. A., and Buratowski, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12542–12547
23. Sherman, F., Fink, G. R., and Hicks, J. B. (1986) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Albertini, M., Pemberton, L. F., Rosenblum, J. S., and Blobel, G. (1998) J. Cell Biol. 143, 1447–1460
25. Rosenblum, J. S., Pemberton, L. F., and Blobel, G. (1997) J. Cell Biol. 139, 1655–1661
26. Sedorf, M., and Silver, P. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8590–8595
27. Lob, J. D., Schlenstedt, G., Pellman, D., Kornitzer, D., Silver, P. A., and Fink, G. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7647–7651
28. Aitchison, J. D., Blobel, G., and Rout, M. P. (1996) Science 274, 624–627
29. Pemberton, L. F., Rosenblum, J. S., and Blobel, G. (1997) J. Cell Biol. 139, 1645–1653
30. Gharahdaghi, F., Kirchner, M., Fernandez, J., and Mische, S. M. (1996) Anai. Biochem. 233, 94–99
31. Eng, J., McCormack, A. L., and Yates, J. R. (1994–1995) Am. Soc. Mass Spectrom. 5, 976–989
32. Yates, J. R. D., Eng, J. K., McCormack, A. L., and Schietz, D. (1995) Anal. Chem. 67, 1426–1436
33. Baake, M., Doncheva, A., and Albig, W. (2001) J. Cell. Biochem. 81, 333–346
34. Schaap, P. J., van't Riet, J., Woldringh, C. L., and Ruus, H. A. (1991) J. Mol. Biol. 221, 225–237
35. Muhlhau瑟, P., Muller, E. C., Otto, A., and Kutay, U. (2001) EMBO J. 20, 690–696
36. Breeuwer, M., and Goldfarb, D. S. (1999) Cell 90, 999–1008
37. Langer, T. (2000) Histochem. Cell Biol. 113, 455–465
