Journal of Cellular Biochemistry 64:573–578 (1997)

Nuclear Transport of H1 Histones Meets the Criteria of a Nuclear Localization Signal–Mediated Process

M. Kurz, D. Doenecke,* and W. Albig

Institute of Biochemistry and Molecular Cell Biology, Georg-August-Universität Göttingen, Göttingen, Germany

Abstract We have studied the nuclear transport of H1 histones using the digitonin permeabilization assay system in order to establish the transport requirements for H1 translocation to the nucleus. Using HeLa cells and fluorescence-labeled calf thymus H1, we show that the H1 nuclear transport in permeabilized cells requires the addition of cytoplasmic extract. Furthermore, it can be blocked by energy depletion and by chilling or by addition of wheat germ agglutinin or by nonhydrolyzable GTP analogs. Thus, the import of H1 histones follows the criteria established for nuclear import mediated by nuclear localization signals (NLS). The distribution of basic amino acids in average H1 sequences, however, does not allow the assignment of a specific element as a classical NLS. J. Cell. Biochem. 64:573–578. © 1997 Wiley-Liss, Inc.

Key words: histone H1; nuclear transport; permeabilized cells; nuclear localization signal

Histones are the major protein constituents of eukaryotic chromatin. Most histone species are synthesized during the S phase of the cell cycle upon chromatin replication [for review see Osley, 1991]. During that period, vast amounts of histone proteins need to be coordinately imported into the nuclear compartment. Since histones are comparatively small molecules with a molecular mass between 11 and 23 kDa [for review see Wolffe, 1995], they might be expected to reach the nuclear compartment by diffusion through the nuclear pore system. However, it has been shown by microinjection of H1 histone into the cytoplasm of *Xenopus laevis* oocytes [Dingwall and Allan, 1984] and PtK1 cells [Breeuwer and Goldfarb, 1990] that H1 histones enter the cell nucleus by a facilitated process that precludes their diffusion through the nuclear pores.

It is still not proven whether H1 histone sequences contain a proper nuclear localization signal (NLS), which mediates the nuclear translocation of newly synthesized H1. Two types of NLS have been identified in several proteins. First, a basic heptapeptide in the SV40 large T-antigen was described as a nuclear transport signal [Kalderon et al., 1984]. A second type of NLS was described in the nucleosome assembly factor nucleoplasmin [Robbins et al., 1991]. This nucleoplasmin NLS is bipartite, consisting of a basic dipeptide followed by 10–12 residues with no apparent bias for certain amino acids and then by at least three basic amino acids within the next five residues [for review see Boulikas, 1994]. Since H1 histones are a lysine-rich class of proteins, several potential NLS motifs may be identified in either the N- or C-terminal domains of the protein.

We have used the permeabilized cell assay system developed by Adam et al. [1990] to study the H1 import characteristics. This method uses digitonin to permeabilize the plasma membrane without affecting the nuclear envelope. As a consequence, the cell can be depleted of cytoplasm, which can then be replaced by an import solution containing the protein to be transported together with cytoplasmic extracts, cytoplasmic subfractions, or purified factors involved in the transport process.

Our results show that the permeabilized cell assay is also suitable for H1 import studies. The data further indicate that the nuclear H1 import meets all criteria of an NLS-mediated transport. Thus, our data serve as a basis for a

Contract grant sponsor: Deutsche Forschungsgemeinschaft (DFG).

*Correspondence to: D. Doenecke, Institute of Biochemistry and Molecular Cell Biology, Georg-August-Universität Göttingen, Humboldtallee 23, D-37073 Göttingen, Germany.

Received 13 September 1996; Accepted 7 October 1996

molecular analysis of such motifs in the H1 molecules which may be involved in this transport process.

METHODS

Cell Culture

HeLa S3 cells were obtained from the American Type Culture Collection (Rockville, MD). They were grown in Modified Eagle's Medium (MEM) (Biochrom, Berlin, Germany) containing 10% fetal calf serum (FCS) (Biochrom) and 1% (w/v) penicillin/streptomycin (BRL, Gaithersburg, MD). Cultures were maintained in a humidified incubator with 5% CO_2 at 37°C. Cells were removed from plastic culture dishes by trypsinization and were replated at a density of $6 \cdot 10^5$ cells/ml on glass coverslips 48 h before use.

In Vitro Transport Assay

Nuclear import was studied using permeabilized HeLa cells [Adam et al., 1990] essentially as described by Moore and Blobel [1992]. HeLa cells were permeabilized for 5 min with 40 µg/ml digitonin (Calbiochem, San Diego, CA) in transport buffer (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, 1 µg/ml each of aprotinin, leupeptin, and pepstatin). Subsequently, the cells were kept for 15 min on ice for efficient cytosol depletion and were washed with transport buffer every 5 min. Transport incubations contained 1 µl 20 mg/ml bovine serum albumin (BSA), 1 µl 100 mM phosphocreatine, 1 µl creatine kinase (400 U/ml), 1 µl 20 mM ATP, 10 µl HeLa cell cytosolic extract (5–10 mg protein/ml, prepared as described by Adam et al. [1990]) and 1 µl import substrate (100–200 nM). The final total volume was adjusted to 20 µl with transport buffer. The import assay was done by incubating the coverslips carrying the permeabilized cells with the import reaction mixture for 30 min at 37°C. After extensive washing with transport buffer, the cells were fixed in 3% formaldehyde for 15 min on ice. Washing, mounting and visualization of the tetramethylrhodamine isothiocyanate (TRITC)-labeled histones was done as described [Moore and Blobel, 1992].

Inhibition experiments were done as indicated either by adding 1 µl 20 mM GTPγS or GMP-PNP (both from Boehringer, Mannheim, Germany) in the presence or absence of ATP or by excluding ATP and its regenerating system or by addition of 1 µl apyrase (2,000 U/ml) (Sigma, St. Louis, MO) and incubation on ice for 15 min. For wheat germ agglutinin (WGA) treatment, permeabilized cells were preincubated with 50 µg/ml WGA (Calbiochem) for 15 min at room temperature.

Transport Substrates

As a control substrate to monitor the transport capacity of the system, TRITC-labeled human serum albumin (HSA) was coupled to a peptide containing the SV40 large T-antigen wild-type (CYTPPKKRKV) or mutant (CYPPKTRKV) NLS [Moore and Blobel, 1992]. For monitoring the import of H1 histones into nuclei of permeabilized cells, calf thymus H1 histones (obtained from Boehringer) were labeled with TRITC. For that purpose, 0.5 mg calf thymus histone H1 was dissolved in 0.5 ml sodium carbonate/sodium bicarbonate, pH 9.0, and allowed to react with 50 µg TRITC (Sigma) for 3 h at room temperature. TRITC-modified histones were purified by gel filtration on Sephadex G25 (Sephadex®; Pharmacia, Uppsala, Sweden). Labeled proteins were stored in aliquots at −20°C and were spun at 10,000 rpm for 10 min prior to use. The transport assay was done as described above, and TRITC-labeled protein was detected by fluorescence microscopy. Nucleoplasm was visualized by the DNA binding substance Diamino-2-phenylindol (DAPI) (Sigma), which was part of the embedding medium [Moore and Blobel, 1992].

RESULTS

Nuclear Transport of H1 Histones

We have used the permeabilization method of Adam et al. [1990] in order to define the general parameters involved in the nuclear transport of H1 histones. HeLa S3 cells were permeabilized with digitonin, and the integrity of the nuclear membrane was tested with control substrates. A digitonin concentration of 40 µg/ml proved to be optimal for the import studies with HeLa cells. At this concentration, the cell morphology remained unchanged, and nuclear import of the control substrate (human serum albumin coupled with a SV40 large T-antigen NLS) was observed (data not shown).

Fluorescence-labeled calf thymus H1 histones were used as transport substrate. This approach was chosen instead of an immunologi-

cal assay with H1 antibodies, since both endogenous HeLa histone H1 and exogenous H1 as transport substrate would react with H1-specific antibodies. Hence, monitoring of the nuclear H1 import would be impossible.

The calf thymus H1 histones were labeled by covalent modification with TRITC. The extent of TRITC labeling was monitored as the quotient of the absorption at 575 nm (TRITC) vs. 280 nm (protein) of the labeled substrate. We observed a dependence of the transport capacity of the system on this degree of labeling, since only transport substrate with a quotient (575:280 nm) of 0.3–0.4 was efficiently transported. Extensively labeled H1 histones with a quotient greater than 0.6 failed to be imported in our assay. This effect may be due to the mode of binding of TRITC which is coupled to amino groups of amino acid side chains. Thus, masking of lysine residue motifs may abolish the nuclear H1 transport through modification of its putative NLS.

The cytoplasmic extract was prepared from HeLa S3 cells grown in suspension and from *Xenopus laevis* oocytes. Since there was no difference in the transport behavior of these two extracts, we used just the HeLa extract in the studies described below. The concentration of the cytoplasmic proteins in the extracts was 4–8 mg/ml. Within this concentration range, we observed no difference in transport capacities. ATP in the transport mixture was regenerated with the phosphocreatine/creatine kinase system. Each set of transport experiments included a series with control substrates. As a positive control for the transport capacity of the cytoplasmic extract and the preparation of the permeabilized cells, TRITC-labeled HSA coupled with an oligopeptide containing the SV40 large T-antigen NLS was used. For testing the integrity of the nuclear membrane, a mutated SV40 large T-antigen NLS was coupled to TRITC-labeled HSA (negative control; control data not shown). The number of coupled oligopeptides was estimated as six to ten by retardation in SDS-polyacrylamide gels.

TRITC-labeled calf thymus H1 histones were efficently imported into the nucleus under standard conditions: cytoplasmic extract, ATP regenerating system, and 37°C incubation temperature (Fig. 1A,B). Replacement of the cytoplasmic extract by transport buffer in the transport assay under otherwise unchanged conditions caused a general disappearance of fluorescence staining, indicating a total loss of transport capacity (Fig. 1C,D). This shows that cytoplasmic components are involved in the import of H1 histones into the nucleus. Thus, despite the comparatively small size of the transport substrate (23 kDa of H1 and about 2 kDa of TRITC residues), H1 is not simply imported by diffusion, but the transport apparently depends on cytoplasmic factors.

When the temperature was lowered from 37°C to 4°C, no fluorescence was detected within the nucleus after the standard incubation time of 30 min (Fig. 1E,F). The fluorescence was detectable only in the cytoplasm and at the nuclear membrane. This absolute dependence on temperature further indicates that the histone H1 nuclear import is not accomplished by passive diffusion but that this transport is a facilitated process.

The analysis of the energy dependence of the nuclear H1 import was done by omitting the ATP regenerating system and by adding apyrase to the cytoplasmic extract in order to deplete the import assay mixture from endogenous ATP. As shown in Figure 1K,L, this energy-depleted system was not capable of facilitating histone H1 import. Again, fluorescence was detectable only in the cytoplasm and at the nuclear envelope.

Further criteria for the facilitated transport of NLS-bearing nuclear proteins include the inhibition of the translocation by preincubation of the permeabilized cells with WGA. WGA binds to the sugar residues of some nuclear pore proteins and thereby abolishes the protein translocation through the nuclear pore complex [Newmeyer and Forbes, 1988]. The preincubation of the cells also inhibits the nuclear accumulation of the fluorescence-labeled H1 histone within the nucleus (Fig. 1G,H). The fluorescence was again localized in the cytoplasm and at the periphery of the nuclear membrane, as in the chilling and energy depletion studies.

The nuclear transport of karyophilic proteins depends on the Ras-related GTPase Ran/TC4 [Moore and Blobel, 1993; Melchior et al., 1993]. The dependence of the histone H1 transport into the nucleus was tested by addition of the nonhydrolyzable GTP analogs GTPγS and GMP-PNP to the transport mixture. The results obtained upon addition of GMP-PNP are shown in Figure 1 I,J. Once again the result is the same as for NLS containing karyophilic proteins. It can be excluded that the inhibitory

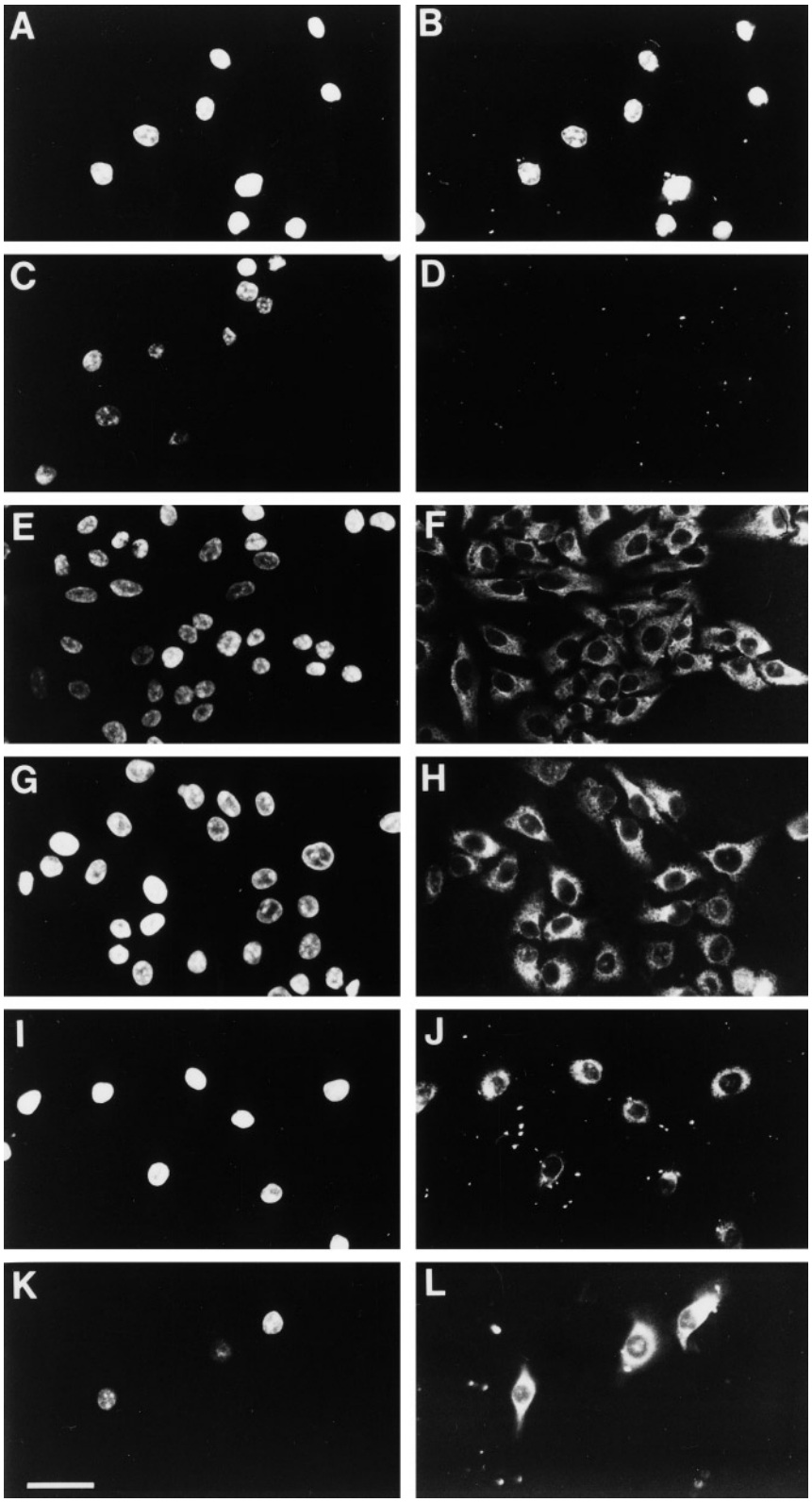

Fig. 1. Nuclear transport of H1 histones: direct fluorescence microscopy of TRITC-labeled calf thymus H1 histones in digitonin-permeabilized HeLa cells (**right column**) compared with DAPI-stained nuclei (**left column**). **A,B:** Permeabilized HeLa cells incubated with TRITC-labeled calf thymus H1 and cytoplasmic extract at 37°C for 30 min. **C,D:** As in A,B, cytoplasmic extract replaced by transport buffer. **E,F** As in A,B but at 4°C. **G,H:** As in A,B but in the presence of wheat germ agglutinin. **I,J:** As in A,B but in the presence of GMP-PNP. **K,L:** As in A,B, but HeLa cells were preincubated with apyrase. Bar, 10 µm.

effect of the nonhydrolyzable GTP analog is due to a depletion of energy, since ATP and the ATP regenerating system were present throughout this assay.

In conclusion, the results of the histone H1 nuclear import studies based on the permeabilization assay prove that this transport depends on a system which meets all the criteria of the NLS-mediated transport of other karyophilic proteins.

DISCUSSION

Several methods have been used to study the nuclear transport of karyophilic proteins. Microinjection of proteins or protein-coated particles into the cytoplasm of somatic cells [Feldherr et al., 1984; Sugawa et al., 1985] or *Xenopus* oocytes [Dingwall et al., 1982], transfection with plasmids encoding proteins with wild-type or mutated NLS motifs [Robbins et al., 1991], and detergent-mediated permeabilization of tumor cells [Adam et al., 1990] or yeast cells [Schlenstedt et al., 1993] have been used for that purpose. Microinjection, which has been used in histone import studies [Dingwall and Allan, 1984; Breeuwer and Goldfarb, 1990], has the advantage of essentially maintaining the integrity of the cells studied. On the other hand, the permeabilized cell assay offers the opportunity to vary the composition of the transport medium and to identify the individual components of a given system. Thus, our data, which extend the results of Breeuwer and Goldfarb [1990], establish the criteria for the analysis of the nuclear import of H1 histones in the permeabilized cell assay system and thus provide the basis for a determination of factors involved in the nuclear import of H1 histones.

H1 histone has a size of 20–23 kDa, depending on the subtype. Initially, molecules smaller than 45 kDa were considered to diffuse freely through the nuclear pore complex [Dingwall et al., 1982], but it has been shown that also the import of several smaller nuclear proteins can depend on a facilitated transport system [Michaud and Goldfarb, 1993]. This import machinery involves several cytoplasmic factors [reviewed in Melchior and Gerace, 1995] including the NLS receptor protein complex [Görlich et al., 1994, 1995; Adam and Adam, 1994], which is the first binding site along the nuclear translocation process.

Breeuwer and Goldfarb [1990] showed by microinjection of histone H1 into the cytoplasm of PtK1 cells that the H1 nuclear transport is arrested in chilled or energy-depleted cells. Imamoto et al. [1995] found that incubation of cytosol with H1-conjugated Sepharose resulted in depletion of the transport capacity for SV40 large T-antigen peptide–NLS conjugates. These data were the basis for the assumption that the H1 transport is an NLS-mediated process. Our data using the permeabilization assay prove that the import of H1 in fact meets all criteria established for an NLS-mediated transport: 1) it depends on the addition of cytoplasmic extract, 2) the transport is energy dependent, 3) it is inhibited by chilling, and 4) it is blocked by GTP analogs. In contrast to other karyophilic proteins, however, in H1 a putative NLS motif is not easily defined since H1 histones are lysine-rich molecules with clusters of lysines in the N- and C-terminal segments of the H1 molecules. Thus, several sequence elements may be considered as potential nuclear localization signals.

H1 has a three-domain structure. The highly conserved central domain is globular and contains just few basic amino acids. The N-terminal domain is free of basic residues in its N-terminal half (about 15 amino acids) but is enriched in basic amino acids in its second half abutting the central domain. In none of the different H1 subtypes, however, do more than three basic residues occur sequentially (e.g., see the human H1 histone subtypes [Albig et al. 1991; Eick et al., 1989; Doenecke and Tönjes, 1986]. The C-terminal domains of all known H1 subtypes are characterized by short repeats of peptide sequences including one or two lysine residues. Thus, combinations of such peptide motifs may serve as bipartite NLS elements in analogy to the nucleoplasmin NLS.

The NLS dependence of the nuclear H1 import, however, does not necessarily imply that H1 histones directly interact with the NLS receptor of the transport system. Alternatively, H1 may interact with transport protein(s) which itself may provide the NLS for the H1 transport towards the nucleoplasm. This possibility would be compatible with any data indicating an NLS participation in H1 nuclear transport, and it would allow for overcoming an inherent problem of the nuclear transport of this highly basic class of proteins. Multigner et al. [1992] have demonstrated that H1 histones tightly bind to microtubuli. Hence, a system shielding newly synthesized H1 from interaction with this or other components of the cytoskeleton may be needed. Thus, an H1 binding protein may serve a dual

function in protecting the newly synthesized karyophilic protein from being trapped outside the nucleus and in mediating the interaction of the H1 molecule with components of the nuclear import system.

As yet, we know only that NLS-mediated transport is involved in the nuclear transport of histone H1. Further studies will show whether the necessary NLS element is provided by the H1 histone itself or by a protein which shields the newly synthesized H1 and guides it to the nuclear import system.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (DFG). Manfred Kurz was the recipient of a fellowship in the DFG graduate program on signal-mediated transport of proteins and vesicles.

REFERENCES

Adam SA, Sterne-Marr R, Gerace L (1990): Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J Cell Biol 111:807–816.

Adam EH, Adam SA (1994): Identification of cytosolic factors required for nuclear location sequence mediated binding to the nuclear envelope. J Cell Biol 125:547–555.

Albig W, Kardalinou E, Drabent B, Zimmer A, Doenecke D (1991): Isolation and characterization of two human H1 genes within clusters of core histone genes. Genomics 10:940–948.

Breeuwer M, Goldfarb DS (1990): Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. Cell 60:999–1008.

Boulikas T (1994): Putative nuclear localization signals (NLS) in protein transcription factors. J Cell Biochem 55:32–58.

Dingwall C, Allan J (1984): Accumulation of the isolated carboxy-terminal domain of histone H1 in the *Xenopus* oocyte nucleus. EMBO J 3:1933–1937.

Dingwall C, Sharnick SV, Laskey RA (1982): A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. Cell 30:449–458.

Doenecke D, Tönjes R (1986): Differential distribution of lysine and arginine residues in the closely related histones H1° and H5. J Mol Biol 187:461–464.

Eick S, Nicolai M, Mumberg D, Doenecke D (1989): Human H1 histones: Conserved and varied sequence elements in two H1 subtype genes. Eur J Cell Biol 49:110–115.

Feldherr CM, Kallenbach E, Schultz N (1984): Movement of a karyophilic protein through the nuclear pore of oocytes. J Cell Biol 99:2216–2222.

Görlich D, Prehn S, Laskey RA, Hartmann E (1994): Isolation of a protein that is essential for the first step of nuclear protein import. Cell 79:767–778.

Görlich D, Kostka, S, Kraft R, Dingwall C, Laskey RA, Hartmann E, Prehn S (1995): Two different subunits of importin cooperate to recognize the nuclear localization signals and bind them to the nuclear envelope. Current Biology 5:383–392.

Imamoto N, Tachibana T, Matsubae M, Yoneda Y (1995): A karyophilic protein forms a stable complex with cytoplasmic components prior to nuclear pore binding. J Biol Chem 270:8559–8565.

Kalderon B, Roberts BL, Richardson WD, Smith AE (1984): A short amino acid sequence able to specify nuclear localization. Cell 39:499–509.

Melchior F, Gerace L (1995): Mechanisms of nuclear protein import. Curr Opin Cell Biol 7:310–318.

Melchior F, Paschal B, Evans J, Gerace L (1993): Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. J Cell Biol 123:1649–1659.

Michaud N, Goldfarb DS (1993): Most nuclear proteins are imported by a single pathway. Exp Cell Res 208:128–136.

Moore MS, Blobel G (1992): The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. Cell 69:939–950.

Moore MS, Blobel G (1993): The GTP binding protein Ran/TC4 is required for protein import into the nucleus. Nature 365:661–663.

Multigner L, Gagnon J, van Dorsselaer A, Job D (1992): Stabilization of sea urchin flagellar microtubules by histone H1. Nature 360:33–39.

Newmeyer DD, Forbes DJ (1988): Nuclear import can be separated into distinct steps in vitro: Nuclear pore binding and translocation. Cell 52:641–653.

Osley MA (1991): The regulation of histone synthesis in the cell cycle. Annu Rev Biochem 60:827–861.

Robbins J, Dilworth SM, Laskey RA, Dingwall C (1991): Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: Identification of a class of bipartite nuclear targeting sequences. Cell 64:615–623.

Schlenstedt G, Hurt E, Doye V, Silver P (1993): Reconstitution of nuclear protein transport with semi-intact yeast cells. J Cell Biol 123:785–798.

Sugawa H, Imamoto N, Wataya-Kaneda M, Uchida T (1985): Foreign protein can be carried into the nucleus of mammalian cell by conjugation with nucleoplasmin. Expte Cell Res 159:419–429.

Wolffe A: (1995): "Chromatin: Structure and Function." London, New York: Academic Press.