MOLECULAR AND CELLULAR BIOLOGY, July 1989, p. 3028–3036
0270-7306/89/073028-09$02.00/0

Vol. 9, No. 7

Identification of Four Nuclear Transport Signal-Binding Proteins That Interact with Diverse Transport Signals

LILI YAMASAKI,[1,2] PATRICK KANDA,[1] AND ROBERT E. LANFORD[1,2]*

Department of Virology and Immunology, Southwest Foundation for Biomedical Research, West Loop 410 at Military Drive, Post Office Box 28147,[1] and Department of Microbiology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive,[2] San Antonio, Texas 78284

Received 2 March 1989/Accepted 17 April 1989

The transport of proteins into the nucleus requires not only the presence of a nuclear transport signal on the targeted protein but also the signal recognition proteins and the nuclear pore translocation apparatus. Complicating the search for the signal recognition proteins is the fact that the nuclear transport signals identified share little obvious homology. In this study, synthetic peptides homologous to the nuclear transport signals from the simian virus 40 large T antigen, *Xenopus* oocyte nucleoplasmin, adenovirus E1A, and *Saccharomyces cerevisiae* MATα2 proteins were coupled to a UV-photoactivable cross-linker and iodinated for use in an in vitro cross-linking reaction with cellular lysates. Four proteins, p140, p100, p70, and p55, which specifically interacted with the nuclear transport signal peptides were identified. Unique patterns of reactivity were observed with closely related pairs of nuclear transport signal peptides. Competition experiments with labeled and unlabeled peptides demonstrated that heterologous signals were able to bind the same protein and suggested that diverse signals use a common transport pathway. The subcellular distribution of the four nuclear transport signal-binding proteins suggested that nuclear transport involves both cytoplasmic and nuclear receptors. The four proteins were not bound by wheat germ agglutinin and were not associated tightly with the nuclear pore complex.

Specific transport of proteins into the nucleus is an important requirement for the integrity of that organelle. Specificity requires the presence of both a nuclear transport signal and the cellular proteins that recognize that signal. Many nuclear transport signals have been defined by mutation and then by their ability as synthetic peptides (15, 22) or as gene fusions (4, 9, 16, 20, 25, 27, 28, 33, 35, 37, 40) to direct nuclear localization of nonnuclear proteins. Unlike transport into the secretory pathway, nuclear transport allows movement without proteolytic removal of the signal, which permits repetitive transport of the protein. These signals are characteristically short amino acid sequences with a high number of basic residues. Variations in the number, the sequence, and the position of the nuclear transport signals have been shown to affect the rate of nuclear uptake (5, 10, 22, 23, 36). There is no consensus nuclear transport signal, which is also the case for transport into the endoplasmic reticulum. Despite the lack of homology and apparent flexibility between nuclear transport signals, decreased transport efficiency occurs when key amino acids are varied (19, 21, 23). This combination of heterogeneity and stringency in nuclear transport signal sequences raises the question of what receptor flexibility and heterogeneity are needed for signal recognition.

Nuclear transport occurs at nuclear pores, which create aqueous, octagonal channels in the double-membraned nuclear envelope (39). Pores restrict the movement of large proteins yet allow free diffusion of small proteins and macromolecules. Electron microscopic studies show that gold particles coated with signal-bearing proteins pass through the nuclear pores of *Xenopus* oocytes (10, 13). The description of in vitro nuclear transport systems has helped further characterize the transport process (18, 26, 29, 31). Wheat germ agglutinin (WGA) binds nuclear pores (14) and pore proteins (6), since many pore proteins have O-linked *N*-acetylglucosamine residues (7, 17, 32, 38). The binding of WGA to nuclear pores is sufficient to inhibit nuclear transport (14), as is an antibody recognizing this O-linked sugar epitope (12). Recently, two groups studying the energy requirements of nuclear transport have proposed that nuclear transport may be divided into two stages, energy-independent binding of proteins to the nuclear envelope and energy-dependent translocation across the nuclear envelope (30, 31, 34). Although the site of entry is at the pore, the initial site for signal recognition has not been defined.

This paper describes the use of synthetic peptides homologous to the nuclear transport signals of the simian virus 40 (SV40) large T antigen, *Xenopus* oocyte nucleoplasmin, adenovirus E1A, and *Saccharomyces cerevisiae* MATα2 proteins for the identification of cellular proteins that bind these transport signals. With the exception of MATα2 conjugates, these same transport peptides functioned as nuclear transport signals when coupled to nonnuclear carrier proteins and microinjected into the cytoplasm of mammalian cells (submitted for publication). In vitro cross-linking studies with UV-activable nuclear transport signal peptides identified four proteins which specifically bound the nuclear transport signals with apparent molecular weights of 140,000, 100,000, 70,000, and 55,000. Two of these proteins, p100 and p70, were cytoplasmic, while the remaining two proteins, p140 and p55, were associated loosely with the nucleus. Competition experiments demonstrated that very different signals were capable of binding the same protein. The proteins did not bind WGA-agarose and did not remain tightly associated with the nuclear pore complex.

MATERIALS AND METHODS

Peptides and cross-linker modification. Six nuclear transport peptides and one control peptide were synthesized with a glycyl-Merrifield resin with a Biosearch 9500 automated

* Corresponding author.

peptide synthesizer. Purification of peptides by reversed-phase high-pressure liquid chromatography was followed by amino acid analysis to verify the composition. Peptides (1.7 μmol) were reduced with dithiothreitol in 50 mM sodium phosphate buffer (pH 7) and then separated from dithiothreitol by P-2 gel filtration (Bio-Rad Laboratories). Peptide fractions were detected by A_{275} and pooled. A fivefold molar excess of the UV cross-linking reagent *para*-azido-phenacylbromide (APB; Pierce Chemical Co.) at 5 mg/ml in methanol was added to the reduced peptide pool. The light-sensitive solution was reacted overnight and then concentrated under vacuum. After removal of a precipitate, the reaction mixture was fractionated on another P-2 column. APB-peptide conjugates had an approximate A_{298}/A_{275} ratio of 1, while free APB had an A_{298}/A_{275} ratio of 1.75. APB-peptide conjugates and free peptides were not well resolved by this method. Ten-nanomole portions of APB-peptide conjugates were iodinated with 0.5 mCi of $Na^{125}I$ and chloramine T. Iodinated peptides were separated from free iodine by P-2 filtration. Specific activities ranged from 12×10^6 to 45×10^6 cpm/nmol of peptide.

Cell fractionation. Cultured buffalo rat liver cells, BRL3A (ATCC CRL-1442), were passaged twice weekly in Ham's nutrient mixture F-12 (Hazelton) with 5% fetal bovine serum and gentamicin. The cells were scraped and pelleted into cold phosphate-buffered saline. After the cells were swelled in hypotonic buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 6.2], 10 mM NaCl, 4 mM $MgCl_2$) for 15 min on ice, they were lysed by 15 strokes in a Dounce homogenizer. The nuclei were pelleted for 10 min at $200 \times g$ at 4°C. The supernatant containing both the cytoplasmic and plasma membrane fractions was clarified further at $3,000 \times g$ at 4°C.

Rat liver nuclei were prepared by the method of Blobel and Potter (3). One A_{260} unit is equal to approximately 3×10^6 nuclei. Nuclei were salt extracted with either 0.15 or 1.0 M NaCl in 50 mM sodium phosphate buffer (pH 7) at 50 U/ml on ice for 15 min. The suspension was vortexed and spun for 3 min in a cold microcentrifuge, which yielded supernatant and pellet fractions. Nuclear pore complex-lamina fractions were prepared by DNase-RNase treatments as previously described (1, 6, 11). Briefly, rat liver nuclei were treated twice with DNase-RNase to produce crude nuclear envelopes. Three equivalent samples of nuclear envelopes were further treated with 2% Triton X-100 or 0.5 M NaCl or both detergent and salt to produce nuclear pore complex lamina fractions. Pellets from each step were used for cross-linking reactions.

In vitro cross-linking. Cross-linking reactions were performed routinely with either cytoplasm from approximately 4.5×10^6 cells or with 1.5 U of rat liver nuclei in 24-well tissue culture dishes (Corning Glass Works). Samples were mixed with 1×10^6 to 2×10^6 cpm of iodinated APB peptide per well in incubation buffer (2 mM HEPES, 40 mM sodium phosphate (pH 7), 52 mM NaCl, 4 mM $MgCl_2$). In addition to the iodinated WT–APB peptide for the competition experiments, various amounts (0.2 to 5.0 nmol) of free peptide were added per well. Reaction mixtures (0.8 or 1.6 ml) were shaken gently for 1 h at room temperature, diluted with the same buffer to 2.4 ml, and incubated for an additional 10 min. To activate the cross-linker, samples were irradiated with longwave UV light (UVGL-25; UVP, Inc.) for 10 min at a distance of 5 cm. Samples were precipitated with 15% trichloroacetic acid overnight at 4°C and spun for 15 min at 4°C in a cold microcentrifuge. Pellets were washed with methanol, dried, and suspended in electrophoresis sample buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% acrylamide gels. Coomassie blue-stained gels were dried for autoradiography.

WGA binding. A BRL3A cell lysate (9.0×10^6 cells) was cross-linked as described above with iodinated WT-APB peptide and dialyzed for 6 h at 4°C into binding buffer (50 mM Tris hydrochloride [pH 7.5], 100 mM NaCl, 1% Nonidet P-40). Rat liver nuclei (20 U) were extracted with 0.15 M NaCl, cross-linked with iodinated WT-APB peptide, and dialyzed for 6 h at 4°C into the binding buffer. As a control, a 75-cm^2 flask of BRL3A cells was labeled overnight with ^{35}S-Translabel (ICN Pharmaceuticals Inc.) at 83 μCi/ml of F-12 medium supplemented with 2% dialyzed fetal bovine serum and lysed by sonication in binding buffer. All three extracts were clarified for 10 min at $3,000 \times g$, and each was mixed with a 0.05-ml sample of WGA-agarose (Vector Laboratories, Inc.) overnight at 4°C. Unbound proteins were removed in the supernatants. The resins were washed with three times with 1 ml (each) of binding buffer. WGA-bound proteins were eluted with 0.5 ml of 0.2 M *N*-acetylglucosamine in binding buffer at pH 7.5 and then with 0.5 ml of 0.5 M *N*-acetylglucosamine in 100 mM glycine buffer at pH 3.0. Starting lysates, unbound lysates, and *N*-acetylglucosamine-eluted proteins were trichloroacetic acid precipitated for SDS-PAGE and autoradiography.

RESULTS

Synthetic peptides. Six nuclear transport signals and a negative control sequence were produced as synthetic peptides for use in this study (Table 1). The WT and mutant cT peptides from amino acid positions 126 to 132 of the SV40 large T antigen differed only at residue 128, where the cT peptide had a neutral asparagine replacing a basic lysine (22). Initially, four putative nuclear transport signals were identified in the carboxy-terminal tail of the *Xenopus* oocyte nucleoplasmin protein by sequence similarities to the SV40 large T antigen and yeast MATα2 signals (8). Only sequences containing the first two putative signal homologies simultaneously were functional in directing the nuclear transport of fusion proteins (4, 9). The first portion of this minimal signal contained a yeast MATα2-like signal at amino acid positions 155 to 162, Lys-Arg-Pro-Ala-Ala-Thr-Lys-Lys. The second portion of this minimal signal contained the SV40 large T-like signal at amino acid positions 167 to 170, Lys-Lys-Lys-Lys. Two nucleoplasmin peptides, NP1 and NP2, were produced for this study. The NP2 peptide contained only the SV40-like sequence, while the longer NP1 peptide contained both the MATα2-like and SV40-like sequences. The NP1 peptide had one additional amino acid at either end of the minimal sequence proposed by Dingwall et al. (9) yet fewer residues at the carboxy terminus of the sufficient nuclear location signal proposed by Bürglin and De Robertis (4). The E1A peptide consisted of the five carboxy-terminal amino acids from the adenovirus E1A transcriptional activator protein (25) and did not resemble either the SV40-like or the MATα2-like sequence. The M2 peptide contained the 13 amino-terminal residues from the yeast transcriptional repressor for mating type switching, MATα2 (16). The negative control peptide, CON, was derived from the procaryotic DNA polymerase factors τ and γ and had six basic residues, three of which were in a single cluster (24). All peptides had amino-terminal cysteine residues, which provided the sulfhydryl moiety for covalent coupling to the cross-linker. Additional glycine residues acted as spacers, and a tyrosine was included for iodination.

TABLE 1. Synthetic peptides used in in vitro cross-linking reactions

Nuclear transport signal peptide	Sequence[a]
SV40	
WT	Cys-Gly-Tyr-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly-Gly
cT	Cys-Gly-Tyr-Gly-Pro-Lys-Asn-Lys-Arg-Lys-Val-Gly-Gly
X. laevis	
NP1	Cys-Gly-Tyr-Gly-Lys-Arg-Pro-Ala-Ala-Thr-Lys-Lys-Ala-Gly-Gln-Ala-Lys-Lys-Lys-Lys-Gly-Gly
NP2	Cys-Gly-Tyr-Gly-Ala-Lys-Lys-Lys-Lys-Leu-Asp-Gly-Gly
Adenovirus E1A	Cys-Gly-Tyr-Gly-Lys-Arg-Pro-Arg-Pro-Gly-Gly
S. cerevisiae M2	Cys-Gly-Tyr-Gly-Met-Asn-Lys-Ile-Pro-Ile-Lys-Asp-Leu-Leu-Asn-Pro-Gln-Gly
CON[b]	Cys-Gly-Tyr-Gln-Arg-Val-Gln-Gly-Ala-Thr-Lys-Ala-Lys-Lys-Ser-Glu-Pro-Ala-Ala-Ala-Thr-Arg-Ala-Arg-Gly

[a] Sequences homologous to the nuclear transport signals are underlined. Amino-terminal cysteines provided sulfhydryl groups for covalent coupling of peptides to the cross-linker APB. Tyrosines were included for iodination, and additional glycine residues acted as spacers.
[b] Negative control peptide.

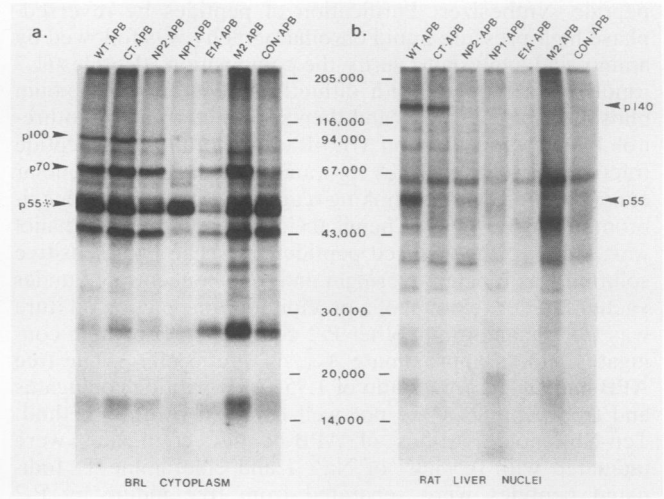

FIG. 1. Four signal-binding proteins identified by cross-linking reactions. In vitro cross-linking reactions with cytoplasm from BRL3A cells (a) and nuclei from rat liver (b) were performed with iodinated nuclear transport signals and negative control peptides modified with the photoactivable cross-linker APB. Panels a and b are autoradiograms of cross-linking reactions after SDS-PAGE with the cytoplasm from 4.5×10^6 cells or 1.5 U of rat liver nuclei loaded per lane, respectively. The sizes of the molecular weight standards used are indicated between the panels. (P55* is a strongly reacting nonspecific protein in the cytoplasmic fraction.)

These signal peptides and the negative control peptide were tested for the ability to induce the nuclear transport of peptide-carrier protein conjugates when microinjected into the cytoplasm of mammalian cells (submitted). All of the signal peptides, except the yeast M2 peptide, were capable of inducing nuclear transport. The CON peptide failed to induce nuclear transport.

Identification and localization of nuclear transport signal-binding proteins. The six nuclear transport peptides and the negative control peptide were coupled to the UV-photoactivable cross-linker APB and labeled with radioactive iodine. To identify proteins that recognize the nuclear transport signals, these modified peptides were incubated in an in vitro cross-linking reaction with either the cytoplasmic fraction of the BRL3A rat liver cell line or nuclei isolated from rat liver tissue. Although nuclei derived from BRL3A cells and rat liver gave similar patterns in cross-linking reactions, the intensity of the signal from rat liver nuclei was stronger. The supernatant fraction from a rat liver tissue homogenate, which contained both a cytoplasmic fraction and ruptured nuclei, was contaminated with blood and connective tissue debris and therefore was not suitable for the cross-linking reaction. For these reasons, BRL3A cytoplasmic fractions and isolated rat liver nuclei were used in these studies. The use of these cellular fractions for identification of several binding proteins does not imply that these proteins reside exclusively in these cellular compartments in vivo. The cross-linking reactions were analyzed by SDS-PAGE and autoradiography. Four proteins that bound the nuclear transport peptides in comparison with the negative control peptide were identified (Fig. 1). The apparent molecular weights of these proteins after cross-linking were 140,000, 100,000, 70,000, and 55,000. Two of the proteins, p100 and p70, appeared in the cytoplasmic supernatant after Dounce homogenization (Fig. 1a) and remained soluble even after ultracentrifugation at 100,000 × *g* for 1 h. The remaining two

proteins, p140 and p55, were most abundant on nuclei (Fig. 1b).

The transport peptides displayed unique patterns of reactivity with the four proteins. Cytoplasmic protein p100 reacted strongly with the WT-APB, cT-APB, and NP2-APB peptides; weakly with the NP1-APB and E1A-APB peptides; and not at all with the M2-APB and CON-APB peptides. Cytoplasmic protein p70 reacted strongly with the WT-APB, cT-APB, NP2-APB, and M2-APB peptides and weakly with the E1A-APB, NP1-APB, and CON-APB peptides. Since protein p70 reacted slightly with the CON-APB peptide, the specificity of this protein for transport signal peptides was questionable. Nuclear protein p140 reacted only with the SV40 large T antigen WT-APB and cT-APB peptides. Nuclear protein p55 reacted exclusively with the SV40 large T antigen WT-APB peptide.

All of the modified transport peptides and the modified negative control peptide additionally recognized a p55* protein in the cytoplasmic fraction. This p55* signal was the only reactivity observed with the iodinated NP1-APB peptide. The reactivity of this p55* protein, however, differed greatly from that of the p55 protein observed on rat liver nuclei. Cytoplasmic protein p55* was bound by all of the peptides, including the negative control peptide. In contrast, nuclear protein p55 was bound only by the WT-APB peptide. The cytoplasmic p55* signal may have been a composite of two or more closely migrating proteins, one with and one without specificity for nuclear transport peptides.

Despite the capacity of the long nucleoplasmin peptide to induce transport in vivo, the iodinated NP1-APB peptide had very little reactivity with any of the four nuclear transport signal-binding proteins. Such discrepancies emphasized the potential biochemical and conformational changes which might have resulted from the APB modification and chloramine T iodination. To circumvent these difficulties, a more sensitive competition assay was developed in which only unmodified, unlabeled peptides were used as competing ligands.

Competition studies. To demonstrate the specificity of the interaction between transport peptides and the nuclear transport signal-binding proteins, a competition assay was developed by using the in vitro cross-linking reaction. The modified SV40 large T antigen WT-APB peptide was used as the iodinated ligand, since this reagent was the only one capable of labeling all four proteins. Unmodified transport and negative control peptides were used as the unlabeled competitors.

With a 0.15 M NaCl supernatant of isolated nuclei, the iodinated WT-APB peptide bound proteins p140 and p55 in the absence of a competitor peptide (Fig. 2). Addition of unlabeled competitor peptides (0.2 nmol per reaction) decreased the labeling of these two proteins. The labeling of protein p140 was competed for strongly by the WT and cT peptides, weakly by the E1A peptide, and not at all by the NP2, M2, and CON peptides. In contrast, labeling of protein p55 was competed for strongly by the WT, NP2, and E1A peptides, weakly by the cT peptide, and not at all by the M2 and CON peptides (the NP1 peptide was not included in this experiment). Labeling of other nonspecific proteins by the iodinated WT-APB peptide was not reduced by addition of transport peptides, which further demonstrated the specificity of the competition reactions. Not only was the binding of the transport peptides to proteins p140 and p55 specific, but the binding proteins also displayed different patterns of reactivity with the transport signals.

These competition studies were expanded to include both

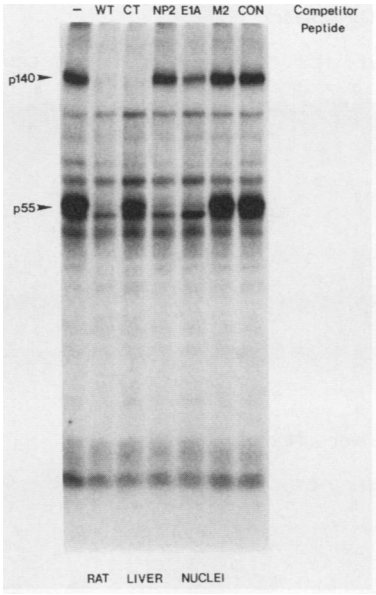

FIG. 2. Competition by transport peptides for iodinated WT-APB binding. A competition assay was developed by using the standard cross-linking reaction with WT-APB as the iodinated ligand and various unmodified peptides as competitors. Reactions with a 0.15 M NaCl supernatant of nuclei were analyzed by SDS-PAGE and autoradiography. Labeling of proteins p140 and p55 decreased dramatically with the addition of different signal peptides.

cytoplasmic and nuclear fractions and two levels (0.5 and 5.0 nmol per reaction) of competitor peptide (Fig. 3; Table 2). The labeling of cytoplasmic protein p100 was decreased by high levels of the WT, cT, NP2, and NP1 peptides and by both high and low levels of the E1A peptide (Fig. 3a). The M2 and CON peptides failed to compete at either level of competitor. The labeling of cytoplasmic protein p70 was decreased by low levels of the NP2, E1A, and M2 peptides but, surprisingly, not by the WT peptide. However, at high levels of competitor peptide, all of the transport peptides, as well as the CON peptide, decreased the labeling of protein p70. The ability of the CON peptide to compete for the labeling of protein p70 raised questions concerning the specificity of this protein for nuclear transport signals.

The labeling of nuclear protein p140 was decreased by both high and low levels of the WT, cT, NP1, and E1A peptides and by only high levels of the NP2 peptide (Fig. 3b; Table 2). The M2 and CON peptides failed to reduce the labeling of protein p140. The competition reaction with the high level of the M2 peptide showed a generalized loss of labeling for all proteins because of a technical problem with this particular reaction and did not reflect genuine competition by the peptide. The labeling of nuclear protein p55 was decreased by high and low levels of the WT, NP2, and E1A peptides and by only high levels of the cT and NP1 peptides. Neither the M2 nor the CON peptide competed for the labeling of protein p55.

A further competition experiment was performed with cytoplasm to evaluate the specificity of transport peptide binding to protein p70 (Fig. 4) by using M2-APB as the iodinated ligand and a subset of unmodified peptides as competitors (0.2 nmol per reaction). Only the E1A and M2 peptides competed, while the WT and CON peptides did not decrease the labeling of protein p70. Therefore, at low levels of competitor peptides, the WT-APB labeling (Fig. 3a) and

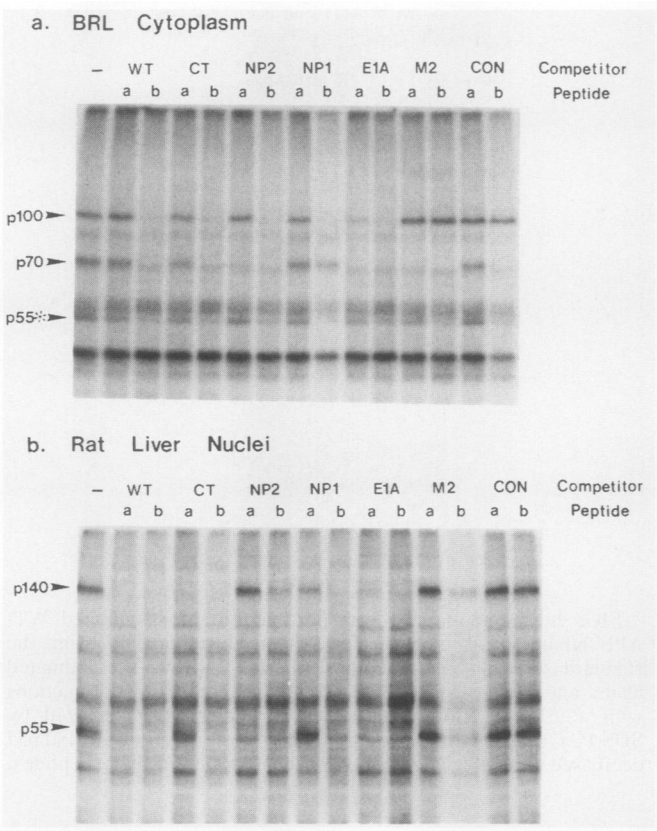

FIG. 3. Distinct competition patterns for each signal-binding protein. Cross-linking reactions with iodinated WT-APB peptide using cytoplasm from 4.5×10^6 cells (panel a) and 1.5 U of nuclei (panel b) were performed with two levels of competitor peptide (lanes a, 0.5 nmol per reaction; lanes b, 5.0 nmol per reaction). The intensities of the four signal-binding proteins fluctuated with the addition of transport peptides but not with addition of the control peptide (see text for the exception, p70). The effectiveness of individual signal peptides to compete varied with the signal-binding protein.

the M2-APB labeling (Fig. 4) of protein p70 were competed for specifically by the same subset of transport peptides. Similar results were obtained with cT-APB or E1A-APB as the iodinated ligand. In all of the experiments, the E1A and M2 signal peptides were the best competitors for protein p70.

Lack of WGA binding to signal-binding proteins. Since WGA inhibits nuclear transport, the WGA-binding potentials of the four signal-binding proteins were examined (Fig. 5). A BRL3A cell lysate containing the p100 and p70 proteins and a 0.15 M NaCl supernatant from rat liver nuclei containing the p140 and p55 proteins were cross-linked with the iodinated WT-APB peptide and then incubated with WGA-agarose samples. Bound proteins were eluted with 0.2 M *N*-acetylglucosamine (pH 7.5) and then with 0.5 M *N*-acetylglucosamine (pH 3.0). The starting material, unbound proteins, and bound protein eluates were analyzed by SDS-PAGE and autoradiography (Fig. 5a and b). All of the signal-binding proteins were detected in the starting extracts and in the unbound fraction but were absent from the *N*-acetylglucosamine eluates. An identical experiment was performed with [^{35}S]methionine-labeled BRL3A cells to demonstrate that proteins were eluted from the resin by *N*-acetylglucosamine. The 0.2 M *N*-acetylglucosamine eluate contained numerous ^{35}S-labeled proteins that bound the WGA resin (Fig. 5c). The profile of the eluted proteins did not resemble the profile of the starting material.

TABLE 2. Competition for [^{125}I]WT-APB labeling

Competitor peptide	Competition[a]			
	p100	p70	p140	p55
WT	+	+	++	++
cT	+	+	++	+
NP2	+	++	+	++
NP1	+	+	++	+
E1A	++	++	++	++
M2	–	++	–	–
CON	–	+	–	–

[a] +, Competition at only high levels of competitor peptide; ++, competition at both low and high levels of competitor peptide; –, no competition at either level of competitor peptide.

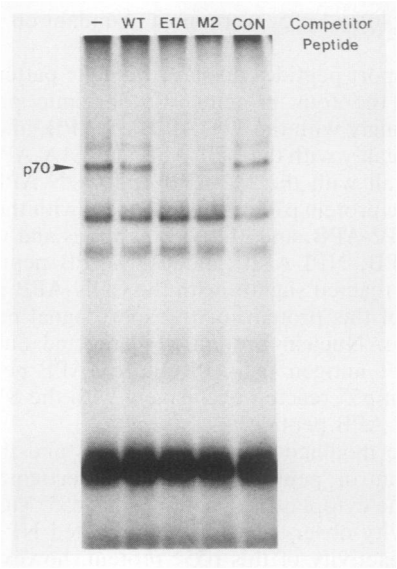

FIG. 4. p70 labeling specifically decreased by low levels of competitor peptide. Cross-linking reactions with iodinated M2-APB peptide and 0.2 nmol of competitor peptide per reaction were performed by using cytoplasm from 4.5×10^6 cells. Reactions were analyzed by SDS-PAGE and autoradiography. E1A and M2 signal peptides specifically competed for the labeling of protein p70, while the WT and CON peptides were poor competitors for this iodinated ligand.

Solubilization of nuclear proteins p140 and p55. Salt fractionation of rat liver nuclei determined how tightly the p140 and p55 proteins were associated with nuclei (Fig. 6). Identical samples of unfractionated nuclei and supernatant and pellet fractions from either a 0.15 M or a 1.0 M NaCl extraction were included in cross-linking reactions with iodinated WT-APB, cT-APB, and CON-APB peptides (Fig. 6, lanes a to c, respectively). As before, the p140 protein was labeled by both the WT-APB and cT-APB peptides, while the p55 protein retained its specificity for the WT-APB peptide. Neither protein was recognized by the CON-APB peptide. Although the p140 and p55 proteins had remained associated with rat liver nuclei, even through a 2.3 M sucrose sedimentation in low-salt buffer during their isolation from tissue, elevation of the buffer to 0.15 M NaCl was sufficient to remove most of the p140 and only a portion of the p55 from the nuclei. The remaining portion of p55 on the nuclei was solubilized by 1.0 M NaCl.

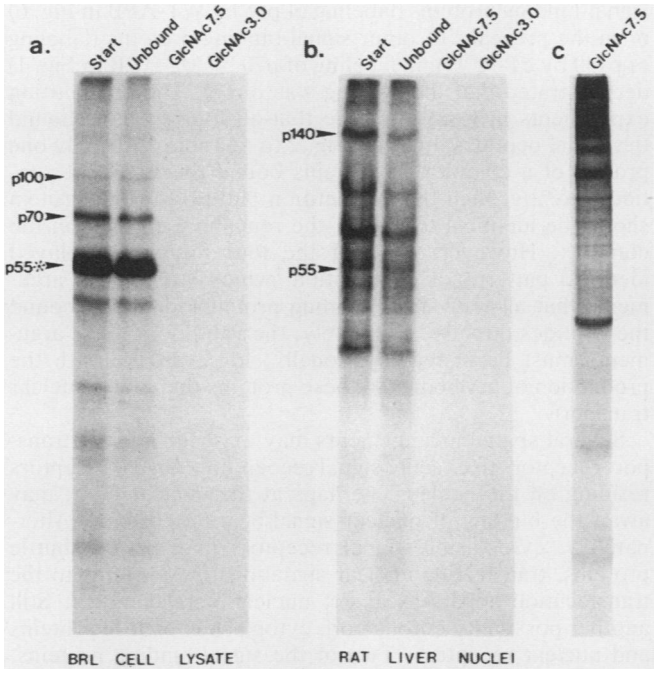

FIG. 5. Lack of WGA-agarose binding. A BRL3A whole-cell lysate (a) and a 0.15 M NaCl extract of nuclei (b) were labeled with iodinated WT-APB peptide and incubated with WGA-agarose. A BRL3A whole-cell lysate labeled with [^{35}S]methionine was also incubated with WGA-agarose (c). The WGA-agarose resins were eluted with *N*-acetylglucosamine (GlcNAc) at pHs 7.5 and 3.0. Samples were subjected to SDS-PAGE and autoradiography. All four signal-binding proteins were found in starting extracts and in the unbound supernatants but not in the *N*-acetylglucosamine eluates. In contrast, many [^{35}S]methionine-labeled proteins were eluted from WGA-agarose under identical conditions (c).

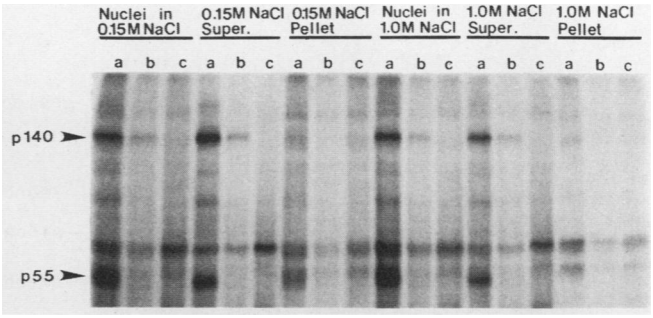

FIG. 6. Salt extraction of nuclear proteins p140 and p55. Rat liver nuclei (12 U) were treated with either 0.15 or 1.0 M NaCl, and half of each sample was centrifuged for 3 min at 16,000 × *g* at 4°C to produce supernatant (Super.) and pellet fractions. Unfractionated nuclei and supernatant and pellet fractions were divided into three identical portions and cross-linked with iodinated peptide WT-APB, cT-APB, or CON-APB (lanes a, b, and c, respectively). The reactions were subjected to SDS-PAGE, and the results were analyzed by autoradiography. Each group of three lanes represents unfractionated nuclei or the supernatant or the pellet from each salt extraction. Extraction of nuclei with 0.15 M NaCl was sufficient to remove protein p140 completely, while 1.0 M NaCl was required to completely solubilize protein p55.

To examine a potential association of the p140 and p55 proteins with nuclear pores, nuclei were fractionated into the nuclear pore complex-lamina and then cross-linked with iodinated WT- and CON-APB peptides (Fig. 7, odd and even lanes, respectively). The isolation procedure involved two DNase-RNase extractions to produce nuclear envelopes (lanes 3 to 6), which were divided into three equal portions for further extraction with 2% Triton X-100 (lanes 7 and 8), 0.5 M NaCl (lanes 9 and 10), or both 2% Triton X-100 and 0.5 M NaCl (lanes 11 and 12), as outlined in Materials and Methods. Cross-linking reactions were analyzed by SDS-PAGE. The Coomassie blue-stained gel demonstrated that similar levels of protein were present in each fraction (Fig. 7a). The autoradiogram of this gel revealed that both nuclear proteins p140 and p55 were solubilized by the first DNase-RNase treatments (Fig. 7b). A low level of p55 reactivity was apparent on nuclear envelopes and crude pore complexes, but four times the amount of nuclear equivalents was required for detection of this reactivity. These results demonstrated that neither protein p140 nor the bulk of protein p55 behaved as a tightly associated nuclear pore protein.

DISCUSSION

The nuclear transport machinery of the cell has at least two functions to perform, nuclear transport signal recognition and protein translocation. Translocation occurs at nuclear pores, which are composed of an array of proteins that regulate the functional size of the channel opening. Signal recognition is provided by the nuclear transport receptors, which are responsible for the specificity of transport and may or may not reside at the site of translocation. Synthetic peptides homologous to known nuclear transport signals from the SV40 large T antigen, *Xenopus* oocyte nucleoplasmin, adenovirus E1A, and yeast MATα2 proteins were used in this study to isolate the transport signal domains from the backbones of these complex, multifunctional proteins. In vitro cross-linking reactions with iodinated transport peptides allowed the identification of cellular proteins that recognized the nuclear transport signals. This study has defined four such proteins, two of which are cytoplasmic proteins, p100 and p70, and two of which are nuclear proteins, p140 and p55.

All of the proteins interacted with at least four of the signal peptides. The transport peptides specifically interacted with these four proteins by two criteria. (i) Subsets of the iodinated transport peptides bound each of these proteins, but the negative control peptide did not. (ii) Unmodified transport-active peptides competed for these proteins, but the negative control peptide did not. The specificity of these interactions and the fact that a heterogeneous group of transport signals bound a small set of cellular proteins present a strong argument that these four proteins are indeed nuclear transport signal receptors. Protein p70 was the only signal-binding protein slightly labeled by the iodinated CON-APB peptide and also competed for by high levels of the unmodified CON peptide. However, at low levels of competitor peptide, the labeling of protein p70 was not decreased by the CON peptide with WT-APB, M2-APB, cT-APB, or E1A-APB as the ligand.

The possibility exists that the nuclear transport signal peptides bound only a subset of the four proteins yet labeled all four proteins by the proximity of the APB cross-linking moiety to a neighboring protein. Two types of evidence against this possibility arise from the localization and competition data. The localization data demonstrate that three of four signal-binding proteins bind the APB-modified peptides directly. The labeling of only one of the signal-binding proteins by APB-modified peptides in the absence of other

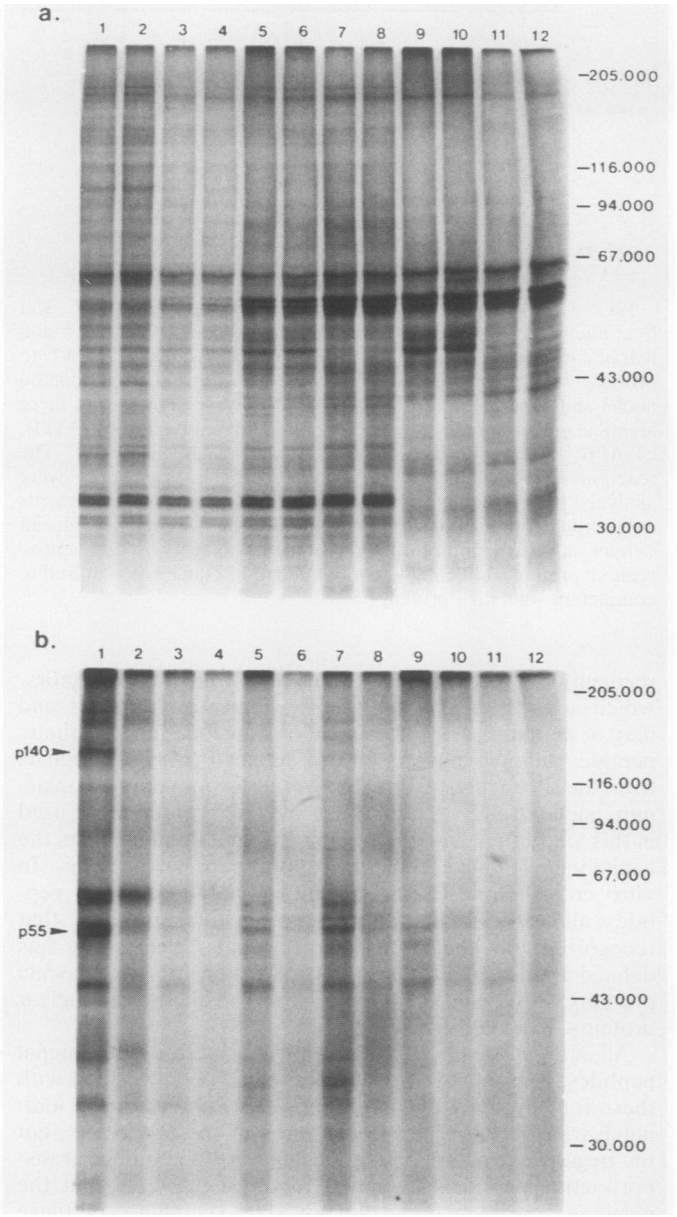

FIG. 7. Proteins p140 and p55 did not fractionate not with nuclear pores. Nuclei were treated twice with DNase-RNase to produce nuclear envelopes, three identical portions of which were extracted further with 2% Triton X-100, 0.5 M NaCl, or both Triton X-100 and NaCl to produce nuclear pore complex-lamina fractions. The pellet from each step was resuspended and divided in half and then cross-linked with either iodinated peptide WT-APB or CON-APB (odd and even lanes, respectively). Samples were subjected to SDS-PAGE and autoradiography. Panel a shows the Coomassie blue-stained gel, and panel b shows the autoradiogram of the fractionation procedure. Lanes: 1 and 2, rat liver nuclei (2 U); 3 and 4, nuclei after the first DNase-RNase treatment (4 U); 5 and 6, crude nuclear envelopes after the second DNase-RNase treatment (8 U); 7 and 8, 2% Triton X-100-extracted nuclear envelopes equivalent to the crude nuclear pore complex-lamina (8 U); 9 and 10, 0.5 M NaCl-extracted nuclear envelopes (16 U); 11 and 12, 2% Triton X-100- and then 0.5 M NaCl-extracted nuclear envelopes (24.5 U). Protein p140 and the bulk of protein p55 were solubilized by the first DNase-RNase treatment. The numbers to the right indicate the sizes of the molecular weight standards used.

signal-binding proteins (labeling of p55 by WT-APB in Fig. 6) or in the presence of other signal-binding proteins (labeling of p140 by cT-APB and labeling of p70 by M2-APB in Fig. 1) demonstrated that the binding was direct. The competition experiments provide evidence that all four proteins bound the signal peptides directly (Fig. 2 to 4; Table 2). If only one protein of a complex of proteins bound the transport peptides directly, then the competition pattern for that protein should be identical to that of the remaining proteins in the complex. However, none of the four proteins displayed identical patterns of competition, which strengthens arguments that all four signal-binding proteins identified bound the peptides directly. Ultimately, the validity of these arguments must be tested functionally, for example, with the production of antibodies to these proteins that block nuclear transport.

Several spatial arrangements may exist for nuclear transport receptors to execute signal recognition. Signal receptors residing on the nucleus, perhaps at the nuclear pore, may await the binding of nuclear signal-bearing proteins. Alternatively, cytoplasmic signal receptors may act as shuttle proteins, transferring nuclear signal-bearing proteins to the translocation apparatus at the nuclear pore complex. Still another possibility enrolls both cytoplasmic-shuttle proteins and nuclear receptors. Two of the signal-binding proteins, p100 and p70, appear to be soluble and cytoplasmic, while the remaining two signal-binding proteins, p140 and p55, are nuclear. The subcellular localization of these signal-binding proteins is consistent with the last spatial arrangement. However, since the nuclear and cytoplasmic fractions used in these cross-linking experiments are from two separate sources, the subcellular location of the signal-binding proteins is tentative. All four of the proteins may be associated with the nuclear envelope or even the nuclear pore complex at some time during the transport process. Immunocytochemical localization with specific antibodies will be required to confirm their tentative nuclear and cytoplasmic localizations.

The striking lack of homology between known nuclear transport signals poses an interesting problem for the recognition machinery of a cell. Three possibilities exist for the recognition of diverse transport signals. One energy-inefficient possibility involves a separate receptor for individual nuclear transport signals. Secondly, groups of nuclear transport signals may have separate receptors with limited flexibility in the receptor-binding pocket. Lastly, the various signals may be recognized by a single receptor with a great deal of flexibility which conducts the proteins into the nucleus. The nuclear transport signals used in this study were a combination of highly divergent sequences and pairs of closely matching sequences. These groups of signals identified multiple signal-binding proteins. Three of the four proteins, p140, p100, and p55, bound five signal peptides but did not recognize the M2 signal. The remaining protein, p70, bound three signal peptides, including the M2 signal, compared with the CON peptide (Table 2). Therefore, each signal does not have a separate receptor and distinct groups of signals do not have separate receptors. Instead, the various signals are recognized by receptors with the ability to see multiple signals. Whether these signals bound one site or different sites on each receptor is yet to be resolved. The competition data, however, suggest that if distinct sites exist, they must be conformationally connected, since different signals compete for binding.

The results of this study are especially intriguing in light of the ability of these same peptides to induce nuclear transport

of peptide-carrier protein conjugates in a microinjection assay (submitted). The ability of the WT, E1A, NP1, and NP2 peptides at low peptide-carrier protein ratios to induce transport is consistent with the ability of these peptides to interact with the four signal-binding proteins. The inability of CON to induce transport is also consistent with the inability of this peptide to interact with at least three of the signal-binding proteins. The ineffectiveness of the cT and M2 peptides at low peptide-carrier protein ratios to induce transport cannot be explained merely as the failure of these signals to bind any of the signal-binding proteins, since the cT peptide competed for all of the signal-binding proteins and M2 competed for the p70 protein (Table 2). In contrast to the M2 peptide, the ability of the cT peptide at high peptide-carrier protein ratios to induce transport suggests that the cT signal defect may reside in a lower affinity of the cT peptide for the signal-binding proteins, which is in agreement with the competition data for protein p55. One explanation for the failure of the M2 signal to induce transport despite the ability of the M2 peptide to bind cytoplasmic protein p70 may be the inability of the nuclear signal-binding proteins of rats (p140 and p55) to recognize the yeast signal.

Since WGA inhibits nuclear transport, the WGA-binding potentials of the nuclear signal-binding proteins were examined; however, none of the four proteins bound to WGA-agarose. Reversal of the procedure, WGA binding and then cross-linking, did not change this result. On one occasion, a protein migrating at 140 kilodaltons (kDa) did bind to WGA-agarose but then failed to react specifically with the iodinated WT-APB peptide (data not shown). The lack of WGA binding by the four nuclear transport signal-binding proteins is consistent with the finding of Newmeyer and Forbes (30) that WGA inhibits only translocation and not signal binding to the nuclear envelope.

To determine whether the p140 and p55 proteins were nuclear pore components, nuclei were fractionated into the nuclear pore complex-lamina and then cross-linked with iodinated WT-APB and CON-APB peptides. Both the p140 and p55 proteins fractionated away from the nuclear pore complex-lamina proteins at the first DNase-RNase treatment of the procedure (Fig. 7). The ease with which proteins p140 and p55 were salt extracted is consistent with a peripheral location for these proteins (Fig. 6). Therefore, if any of these nuclear transport signal-binding proteins reside at the pore, they must be very loosely associated nuclear pore proteins.

Recently, two groups have identified antibodies to nuclear proteins which inhibit nuclear transport. A monoclonal antibody, RL1, which recognizes the O-linked *N*-acetylglucosamine modification as part of its immunodeterminant binds directly to the nuclear pore complex and inhibits the transport of nucleoplasmin, 5S rRNA, and tRNA (12). By Western blotting (immunoblotting), RL1 recognizes 210-, 180-, 145-, 100-, 63-, 58-, 54-, and 45-kDa proteins on nuclear envelopes. A polyclonal immunoglobulin G preparation against the negatively charged peptide Asp-Asp-Asp-Glu-Asp also blocks the transport of nucleoplasmin, SV40 large T antigen-bovine serum albumin, and polyomavirus large T antigen-bovine serum albumin peptide conjugates (41). Two nuclear proteins migrating at 69 and 59 kDa were immunoblotted, while five additional proteins at 65, 54, 50, 43, and 34 kDa were immunoprecipitated from high-salt, detergent-treated nuclear envelope extracts with this antibody preparation. Whether any of the four proteins identified by in vitro cross-linking reactions belong to the sets of proteins recognized by these antibodies is yet to be determined. Although both groups have demonstrated inhibition of nuclear transport, the stage at which the antibodies intervene may well be translocation and not signal recognition.

During the preparation of this report, Adam et al. (2) reported the identification of two signal-binding proteins (70 and 60 kDa) by use of SV40 large T antigen peptides for chemical cross-linking. The binding and fractionation properties of the 60-kDa protein appear quite similar to those of protein p55 in this report; however, the properties of the 70-kDa protein appear to be distinct from those of protein p70 in this report. The use of nuclear transport signals other than those from the SV40 large T antigen has facilitated the identification of two additional signal-binding proteins and has addressed the use of the receptors by heterologous nuclear transport signals.

ACKNOWLEDGMENTS

We gratefully acknowledge Raymond G. Dunham and Kevon Shuler for assistance in peptide synthesis and Robert G. White for assistance in evaluation of the in vivo transport efficiency of the peptides.

This investigation was supported in part by Public Health Service research grant CA 39390 from the National Cancer Institute.

LITERATURE CITED

1. **Aaronson, R. P., and G. Blobel.** 1974. On the attachment of the nuclear pore complex. J. Cell Biol. **62**:746–754.
2. **Adam, S. A., T. J. Lobl, M. A. Mitchell, and L. Gerace.** 1989. Identification of specific binding proteins for a nuclear location sequence. Nature (London) **337**:276–279.
3. **Blobel, G., and V. R. Potter.** 1966. Nuclei from rat liver: isolation method that combines purity with high yield. Science **154**:1662–1665.
4. **Bürglin, T. R., and E. M. De Robertis.** 1987. The nuclear migration signal of Xenopus laevis nucleoplasmin. EMBO J. **6**:2617–2625.
5. **Colledge, W. H., W. D. Richardson, M. D. Edge, and A. E. Smith.** 1986. Extensive mutagenesis of the nuclear location signal of simian virus 40 large-T antigen. Mol. Cell. Biol. **6**:4136–4139.
6. **Davis, L. I., and G. Blobel.** 1986. Identification and characterization of a nuclear pore complex protein. Cell **45**:699–709.
7. **Davis, L. I., and G. Blobel.** 1987. Nuclear pore complex contains a family of glycoproteins that includes p62: glycosylation through a previously unidentified cellular pathway. Proc. Natl. Acad. Sci. USA **84**:7552–7556.
8. **Dingwall, C., S. M. Dilworth, S. J. Black, S. E. Kearsey, L. S. Cox, and R. A. Laskey.** 1987. Nucleoplasmin cDNA sequence reveals polyglutamic acid tracts and a cluster of sequences homologous to putative nuclear localization signals. EMBO J. **6**:69–74.
9. **Dingwall, C., J. Robbins, S. M. Dilworth, B. Roberts, and W. D. Richardson.** 1988. The nucleoplasmin nuclear location sequence is larger and more complex than that of SV40 large T antigen. J. Cell Biol. **107**:841–849.
10. **Dworetzky, S. I., R. E. Lanford, and C. M. Feldherr.** 1988. The effects of variations in the number and sequence of targeting signals on nuclear uptake. J. Cell Biol. **107**:1279–1287.
11. **Dwyer, N., and G. Blobel.** 1976. A modified procedure for the isolation of a pore complex-lamina fraction from rat liver nuclei. J. Cell Biol. **70**:581–591.
12. **Featherstone, C., M. K. Darby, and L. Gerace.** 1988. A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA *in vivo*. J. Cell Biol. **107**:1289–1297.
13. **Feldherr, C. M., E. Kallenbach, and N. Schultz.** 1984. Movement of a karyophilic protein through the nuclear pores of oocytes. J. Cell Biol. **99**:2216–2222.
14. **Finlay, D. R., D. D. Newmeyer, T. M. Price, and D. J. Forbes.** 1987. Inhibition of *in vitro* nuclear transport by a lectin that binds to nuclear pores. J. Cell Biol. **104**:189–200.
15. **Goldfarb, D. S., J. Gariepy, G. Schoolnik, and R. D. Kornberg.**

1986. Synthetic peptides as nuclear localization signals. Nature (London) **322**:641–644.
16. **Hall, M. N., L. Hereford, and I. Herskowitz.** 1984. Targeting of E. coli β-galactosidase to the nucleus in yeast. Cell **36**:1057–1065.
17. **Holt, G. D., C. M. Snow, A. Senior, R. S. Haltiwanger, L. Gerace, and G. W. Hart.** 1987. Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked *N*-acetylglucosamine. J. Cell Biol. **104**:1157–1164.
18. **Imamoto-Sonobe, N., Y. Yoneda, R. Iwamoto, H. Sugawa, and T. Uchida.** 1988. ATP dependent association of nuclear proteins with isolated rat liver nuclei. Proc. Natl. Acad. Sci. USA **85**:3426–3430.
19. **Kalderon, D., W. D. Richardson, A. F. Markham, and A. E. Smith.** 1984. Sequence requirements for nuclear location of simian virus 40 large-T antigen. Nature (London) **311**:33–38.
20. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino acid sequence able to specify nuclear location. Cell **39**:499–509.
21. **Lanford, R. E., and J. S. Butel.** 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. Cell **37**:801–813.
22. **Lanford, R. E., P. Kanda, and R. C. Kennedy.** 1986. Induction of nuclear transport with a synthetic peptide homologous to the SV40 T antigen transport signal. Cell **46**:575–582.
23. **Lanford, R. E., R. G. White, R. G. Dunham, and P. Kanda.** 1988. Effect of basic and nonbasic amino acid substitutions on transport induced by simian virus 40 T-antigen synthetic peptide nuclear transport signals. Mol. Cell. Biol. **8**:2722–2729.
24. **Lee, S. H., P. Kanda, R. C. Kennedy, and J. R. Walker.** 1987. Relation of the *Escherichia coli* dnaX gene to its two products—the τ and γ subunits of DNA polymerase III holoenzyme. Nucleic Acids Res. **15**:7663–7675.
25. **Lyons, R. H., B. Q. Ferguson, and M. Rosenberg.** 1987. Pentapeptide nuclear localization signal in adenovirus E1A. Mol. Cell. Biol. **7**:2451–2456.
26. **Markland, W., A. E. Smith, and B. L. Roberts.** 1987. Signal-dependent translocation of simian virus 40 large-T antigen into rat liver nuclei in a cell-free system. Mol. Cell. Biol. **7**:4255–4265.
27. **Moreland, R. B., G. L. Langevin, R. H. Singer, R. L. Garcea, and L. M. Hereford.** 1987. Amino acid sequences that determine the nuclear localization of yeast histone 2B. Mol. Cell. Biol. **7**:4048–4057.
28. **Moreland, R. B., H. G. Nam, L. M. Hereford, and H. M. Fried.** 1985. Identification of a nuclear localization signal of a yeast ribosomal protein. Proc. Natl. Acad. Sci. USA **82**:6561–6565.
29. **Newmeyer, D. D., D. R. Finlay, and D. J. Forbes.** 1986. *In vitro* transport of a fluorescent nuclear protein and exclusion of non-nuclear proteins. J. Cell Biol. **103**:2091–2102.
30. **Newmeyer, D. D., and D. J. Forbes.** 1988. Nuclear import can be separated into distinct steps *in vitro*: nuclear pore binding and translocation. Cell **52**:641–653.
31. **Newmeyer, D. D., J. M. Lucocq, T. R. Bürglin, and E. M. De Robertis.** 1986. Assembly *in vitro* of nuclei active in nuclear protein transport: ATP is required for nucleoplasmin accumulation. EMBO J. **5**:501–510.
32. **Park, M. K., M. D'Onofrio, M. C. Willingham, and J. A. Hanover.** 1987. A monoclonal antibody against a family of nuclear pore proteins (nucleoporins): O-linked *N*-acetylglucosamine is part of the immunodeterminant. Proc. Natl. Acad. Sci. USA **84**:6462–6466.
33. **Picard, D., and K. R. Yamamoto.** 1987. Two signals mediate hormone-dependent nuclear localization of the glucocortocoid receptor. EMBO J. **6**:3333–3340.
34. **Richardson, W. D., A. D. Mills, S. M. Dilworth, R. A. Laskey, and C. Dingwall.** 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell **52**:655–664.
35. **Richardson, W. D., B. L. Roberts, and A. E. Smith.** 1986. Nuclear location signals in polyoma virus large T. Cell **44**:77–85.
36. **Roberts, B. L., W. D. Richardson, and A. E. Smith.** 1987. The effect of protein context on nuclear location signal function. Cell **50**:465–475.
37. **Silver, P. A., L. P. Keegan, and M. Ptashne.** 1984. Amino terminus of the yeast GAL4 gene product is sufficient for nuclear localization. Proc. Natl. Acad. Sci. USA **81**:5951–5955.
38. **Snow, C. M., A. Senior, and L. Gerace.** 1987. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. J. Cell Biol. **104**:1143–1156.
39. **Unwin, P. N. T., and R. A. Milligan.** 1982. A large particle associated with the perimeter of the nuclear pore complex. J. Cell Biol. **93**:63–75.
40. **Wychowski, C., D. Benichou, and M. Girard.** 1986. A domain of SV40 capsid polypeptide VP1 that specifies migration into the cell nucleus. EMBO J. **5**:2569–2576.
41. **Yoneda, Y., N. Imamoto-Sonobe, Y. Matsuoka, R. Iwamoto, Y. Kiho, and T. Uchida.** 1988. Antibodies to Asp-Asp-Glu-Asp can inhibit transport of nuclear proteins into the nucleus. Science **242**:275–278.