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Identification and characterization of a nuclear localization sequence-binding protein in yeast

(nuclear transport/ligand blotting/*Saccharomyces cerevisiae*)

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ABSTRACT **Nuclear proteins contain specific regions that are required for entry into the nucleus. Using ligand blotting, we have shown that a 67-kDa yeast nuclear envelope protein (p67) recognizes synthetic peptides containing the yeast histone H2B or simian virus 40 large tumor antigen nuclear localization sequence. Both free peptide and peptide conjugated to human serum albumin are recognized. The interaction between p67 and the nuclear localization sequences is specific; neither a mutant peptide that is incompetent for nuclear transport *in vivo* nor HSA can interact with p67 on blots. Moreover, although the wild-type peptide competes for binding to p67, the mutant peptides do not. p67 appears to be located at the nuclear envelope and is not present in other subcellular fractions. The nuclear localization sequence-binding protein is not extracted from the nuclear envelope with nonionic detergents and only partially extracted with high-salt buffer or 8 M urea, suggestive of a tight association with the nuclear envelope. Together our results are consistent with a role for p67 in nuclear transport.**

Studies using dextrans and small nonnuclear proteins have shown that the nuclear envelope is a molecular sieve for proteins the size of 20–40 kDa (1). Evidence suggests that nuclear proteins larger than 40 kDa are actively transported into the nucleus by way of the nuclear pores (see reviews in refs. 2 and 3). The transport of nuclear proteins has been shown to occur in two steps: binding and subsequent translocation. Only the translocation step requires ATP (4–7). Endogenous nuclear localization sequences have been shown to direct nuclear proteins to the nucleus and, through gene fusions, can also mediate the transport of cytoplasmic and bacterial proteins into the nucleus (see reviews in refs. 2 and 3). In addition, synthetic peptides containing the nuclear localization sequence of simian virus 40 large tumor antigen (SV40 T antigen), when chemically conjugated to nonnuclear proteins, are also capable of targeting them to the nuclear compartment (8–10).

Recently, two different investigators have used chemical cross-linking methods in rat liver to show that proteins in the cytoplasm and at the nuclear envelope specifically interact with synthetic peptides containing nuclear localization sequences. These are the first candidates for proteins likely to be involved in the initial signal-recognition step required for nuclear transport. Using a synthetic SV40 T-antigen peptide, Adam *et al.* (11) found a major 60-kDa and a minor 70-kDa protein that were distributed primarily in the cytoplasm but also in the nuclear interior and nuclear envelope. The authors suggest a multistep model for nuclear protein transport in which a cytoplasmic receptor binds to a nuclear protein and carries the protein into the nucleus via a second receptor at the nuclear envelope. Prompt dissociation within the nucleus would then lead to recycling of the receptor to the cytoplasm. Yamasaki *et al.* (12) also reported additional proteins that recognize a number of heterologous synthetic nuclear localization sequences. Two of the proteins, p100 and p70, are located in the cytoplasm, and two others, p140 and p55, are loosely associated with the nuclear envelope.

In this study, we used synthetic peptides containing nuclear localization sequences to search for nuclear localization sequence-binding protein(s) at the nuclear envelope of yeast. The technique of ligand blotting was used to identify a nuclear envelope protein (p67) that specifically interacts with two different synthetic peptides containing the nuclear localization sequences of histone H2B or SV40 T antigen. The specific binding and the presence of p67 in nuclei and nuclear envelopes is consistent with it having a role in nuclear transport. This protein has different biochemical characteristics from the proteins previously identified in rat liver; it is not present in the cytoplasmic fraction and appears to be tightly associated with the nuclear envelope.

MATERIALS AND METHODS

Strains and Subcellular Fractionation. The protease-deficient haploid *Saccharomyces cerevisiae* strain BJ 2168 (**a**, *trp1*, *leu2*, *ura3-52*, *prb1-1122*, *prc1-407*, *pep4-3*) was obtained from the Yeast Genetic Stock Center (University of California, Berkeley, CA). Yeast cells were lysed and fractionated, and the nuclei were isolated by the method of Aris and Blobel (13). For those experiments where a cytosolic fraction was required, we either used the soluble fraction (S) from the above nuclear isolation protocol or cytosol prepared by a different method specifically developed for isolating pure cytoplasm (14). Nuclear envelopes were generated by digesting purified nuclei with DNase I as described (13). DNase I supernatant was further cleared by centrifugation at 356,000 × *g* for 1 hr. Extractions were carried out by resuspending nuclear envelopes in various extracting reagents (1% Triton X-100, 0.25 M KCl, 2% Triton X-100/2 M KCl, and 8 M urea/4 mM EDTA; all of the extracting buffers contained 10 mM Tris·HCl, pH 7.0) at room temperature for 10 min and then centrifuging them in a Beckman TLA 100.2 rotor at 356,000 × *g* for 1 hr. The proteins in the supernatants were precipitated with 15% trichloroacetic acid at 4°C for 12 hr. The samples were then prepared for ligand blotting.

Ligand Blotting. Samples were solubilized without boiling in Laemmli sample buffer (15) that also contained 6 M urea and were then applied to a $NaDodSO_4$/10.5% polyacrylamide gel run at 40 mA for 4.5 hr or 12 mA for 12 hr or were applied to a $NaDodSO_4$/19% polyacrylamide gel run at 9 mA for 18 hr. The $NaDodSO_4$/19% polyacrylamide gel was stained with Coomassie blue to visualize the histone proteins. The proteins separated on $NaDodSO_4$/10.5% polyacrylamide gels were electrophoretically transferred to two nitrocellulose

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Abbreviations: SV40 T antigen, simian virus 40 large tumor antigen; HSA, human serum albumin; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester.

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filters (50 V, 3.5 hr) in a buffer containing 0.1% $NaDodSO_4$, 0.19 M glycine, 25 mM Tris base, and 20% (vol/vol) methanol. After transfer, one filter was stained with 0.1% india ink (16) and the other filter was washed with 50% (vol/vol) 2-propanol in water, rinsed with distilled water, and blocked with buffer A (15 mM Tris·HCl, pH 7.3/150 mM NaCl/2 mM $MgCl_2$/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride/0.1% Tween 20/0.1% gelatin) at room temperature for 1 hr (17). The filter was then incubated with buffer A containing the ligands (and the competitors) for at least 18 hr. During this period, the proteins were allowed to renature and bind to the ligands (17). When peptide–human serum albumin (HSA) conjugates were used as ligands, the binding of ligands was detected by immunochemical methods. In most cases rabbit anti-HSA antiserum and peroxidase-labeled goal anti-rabbit IgG (Cappel Laboratories) were used; in some cases, anti-HSA antiserum and ^{125}I-labeled protein A (DuPont) were used (18). When ^{14}C-labeled peptides were used as ligands, the binding was analyzed by exposing the filter to Kodak XAR-5 film in a Cronex cassette containing a Lightning Plus intensifying screen (Picker, Highland Heights, OH) at −80°C.

Peptide Synthesis. The H2B peptides were synthesized by Janis Young at the UCLA Peptide Synthesis Facility (Los Angeles). A 15-mer was made that contained 13 amino acids of the histone H2B sequence, including the nuclear localization sequence (underlined in the sequence below) and two additional residues, a tyrosine for ^{125}I-labeling and a cysteine for conjugation reactions. A mutant H2B peptide was also synthesized that substituted methionine for lysine-31. The SV40 T-antigen peptides, wild-type and mutant, were generously supplied by Tom Meier and Günter Blobel (Rockefeller University). H2B peptides were: wild type, NH_2-Ser-Thr-Asp-Gly-Lys-Lys-Arg-Ser-Lys-Ala-Arg-Lys-Glu-Tyr-Cys-COOH; and mutant, identical peptide except for replacement of Lys-31 by methionine. SV40 T-antigen peptides were: wild type, NH_2-Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Gly-Gly-Tyr-Cys-COOH; and mutant, identical peptide except for replacement of Lys-128 by threonine.

Coupling of Synthetic Peptides to HSA. Peptides were coupled to HSA through the cysteine of the peptide with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS; Sigma) as the coupling reagent (19).

For each peptide, 0.7 mg of MBS in dimethylformamide was added to 4 mg of HSA in 0.25 ml of 10 mM sodium phosphate buffer (pH 7.2) and allowed to shake for 30 min at room temperature. The reaction product was passed through a Sephadex G-25 (Pharmacia) column to remove free MBS. The MBS-activated HSA was then treated with 5 mg of peptide dissolved in 1 ml of phosphate-buffered saline for 3 hr at room temperature. Free peptide was removed by the Sephadex G-25 column.

According to the molecular weight change in $NaDodSO_4$/polyacrylamide gel electrophoresis, it was estimated that each HSA molecule contained 10–20 peptides.

^{14}C-Labeling of Peptides. The synthetic peptides were ^{14}C-labeled by reductive methylation (20, 21); 2 mg of peptide was dissolved in 70 μl of 10 mM potassium phosphate buffer (pH 7.5) and treated with 10 μl of 20 mM $NaCNBH_4$ and 40 μCi of [^{14}C]formaldehyde (ICN; 1 Ci = 37 GBq) for 12 hr. The product was stored at −20°C.

RESULTS

A 67-kDa Protein (p67) at the Nuclear Envelope Binds the Histone H2B Nuclear Localization Sequence. The N terminus of the yeast histone H2B protein has been shown to contain a stretch of seven amino acid residues (Gly-Lys-Lys-Arg-Ser-Lys-Ala) that can direct β-galactosidase to the nucleus *in vivo* (22). These residues resemble the SV40 T-antigen nuclear localization sequence (Pro-Lys-Lys-Lys-Arg-Lys-Val) (23). When a critical lysine residue at position 128 is changed to threonine in the SV40 T-antigen signal (24) or lysine-31 is changed to a methionine in the histone H2B signal (22), the nuclear localization sequences are no longer functional *in vivo*. In addition, the SV40 T-antigen targeting sequence has been shown to direct both a secretory and a bacterial protein to the yeast nucleus *in vivo* (25). We have used a synthetic peptide containing either the nuclear localization sequence of H2B or SV40 T-antigen as well as a mutant H2B (Lys-31 → Met) or SV40 T-antigen (Lys-128 → Thr) peptide to search for a receptor protein at the yeast nuclear envelope that specifically recognizes transport-competent nuclear localization sequences. The wild-type SV40 T-antigen peptide used in this study is capable of directing a nonnuclear protein to the rat liver nucleus *in vitro*, whereas the mutant peptide is not (Tom Meier, personal communication).

We used a technique known as ligand blotting that was designed to detect the binding between proteins bound on nitrocellulose paper and ligands in solution (26). Various ligands can be used—e.g., DNA, RNA, proteins, or small molecules. The method involves separation of proteins by gel electrophoresis, transfer of the separated proteins to a nitrocellulose filter, and analysis of the interaction of the bound proteins with different ligands. Yeast nuclear envelope proteins were separated by $NaDodSO_4$ polyacrylamide gel electrophoresis, and subsequently were blotted to nitrocellulose filters. The proteins were allowed to renature (17) and interact with wild-type H2B peptide-HSA conjugate or ^{14}C-labeled free H2B peptide (as described in *Materials and Methods*). The results are shown in Fig. 1. The binding of the H2B peptide-HSA conjugate was detected by anti-HSA antiserum and either enzyme-labeled (Fig. 1, lanes 1 and 2) or radiolabeled (Fig. 1, lane 3) secondary reagents. Both the free and conjugated peptide interacted with one major protein(s), p67, in the nuclear envelope fraction (Fig. 1, compare lanes 2 and 4). The wild-type H2B peptide bound to a doublet at 67 kDa that was not as evident when peptide conjugates were used as ligands. The doublet may be due to proteolytic degradation or post-

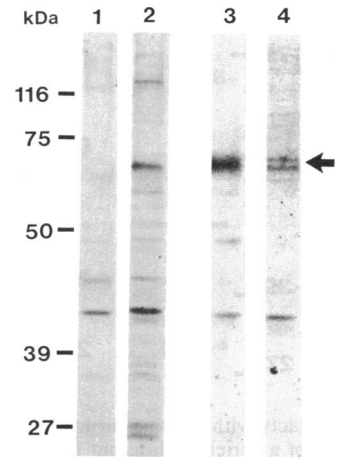

FIG. 1. Ligand blotting of nuclear envelope proteins with wild-type H2B peptide or H2B peptide–HSA conjugates. In lanes 1 and 2, the binding of ligand was detected by an enzyme-labeled secondary reagent. Lanes: 1, control blot incubated with rabbit anti-HSA antiserum and peroxidase-labeled goat anti-rabbit IgG to show the nonspecific binding of antibodies; 2, blot probed with wild-type H2B-HSA conjugate and detected with the same two antibodies used in lane 1; 3, an autoradiogram of a ligand blot probed with wild-type H2B peptide-HSA conjugate followed by detection with anti-HSA antiserum and ^{125}I-labeled protein A; 4, autoradiogram of ligand blot with free ^{14}C-labeled wild-type H2B peptide as the ligand. The arrow shows p67, which appears as a doublet in lane 4.

translational modification or may represent two different proteins; our data cannot distinguish between these alternatives.

Several low molecular weight protein bands were recognized, but these bands appeared nonspecific because: (*i*) they were present in a control blot only incubated with antibodies and no ligands (Fig. 1, lane 1), and (*ii*) the binding of the peptide conjugates to these proteins was not affected by free wild-type H2B peptide (see below). Another protein at ≈120 kDa was not present on control blots and could possibly be another sequence-binding protein, but our data is not sufficient at present to make this statement.

Interaction Between p67 and Nuclear Localization Sequences Is Specific. When wild-type H2B (Fig. 2*A*, lane 2) or SV40 T-antigen peptide-HSA conjugates (Fig. 2*A*, lane 4) were used to probe blots of nuclear envelopes, both peptide conjugates recognized a 67-kDa protein. However, mutant H2B peptide or mutant SV40 T-antigen peptide-HSA conjugates did not bind to p67 (Fig. 2*A*, lanes 3 and 5, respectively). HSA itself also did not recognize p67 in the nuclear envelope (Fig. 2*A*, lane 1).

In competition experiments, the binding of wild-type H2B conjugate to p67 was almost completely abolished when incubated in the presence of a 30-fold excess of free wild-type peptide (Fig. 2*B*, lane 2), while binding was not affected in the presence of free mutant H2B peptide (Fig. 2*B*, compare lanes 1 and 3).

p67 Is Only Found in Nuclei and Nuclear Envelopes. The Ficoll 400 step gradient used to isolate yeast nuclei was prepared by loading a spheroplast lysate in 20% Ficoll over three layers containing 30%, 40%, and 50% Ficoll. After centrifugation, four fractions were analyzed to obtain a representative cross section of the proteins present in the spheroplast lysate. The fractions have been characterized (13) and are designated in Fig. 3 along with the nuclear envelope fraction. The four major fractions contain all of the proteins of yeast with the exception of cell-wall components.

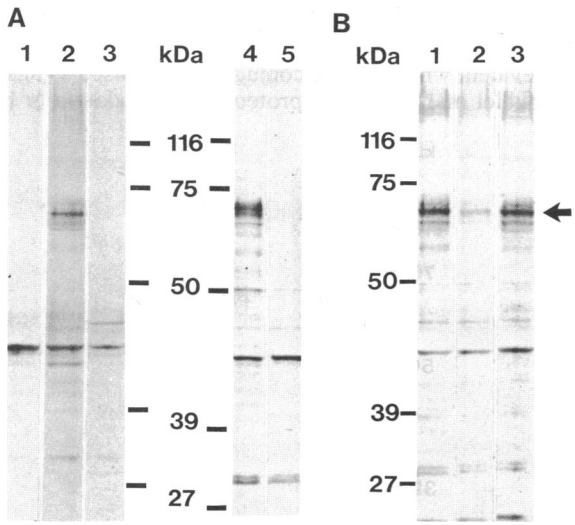

FIG. 2. p67 interacts with the nuclear localization signal specifically. (*A*) Binding of a series of different ligands to nuclear envelopes. Lanes: 1, HSA; 2–5, various peptide-HSA conjugates detected with anti-HSA antibody and peroxidase-labeled secondary antibody [wild-type H2B peptide (lane 2), mutant H2B peptide (lane 3), wild-type SV40 T-antigen peptide (lane 4), and mutant SV40 T-antigen peptide (lane 5)]. Lanes 1–3 and lanes 4 and 5 are from different ligand blots and were electrophoresed under different conditions. (*B*) Competition experiments with wild-type and mutant peptides. Identical amounts (10 μg/ml) of wild-type H2B peptide-HSA conjugate were used in all blots, but different competitors were added. Lanes: 1, no competitor; 2, wild-type H2B peptide (75 μg/ml); 3, mutant H2B peptide (75 μg/ml). Competitions were carried out with a 30-fold excess of free peptide as compared with the coupled peptide. p67 is marked by an arrow.

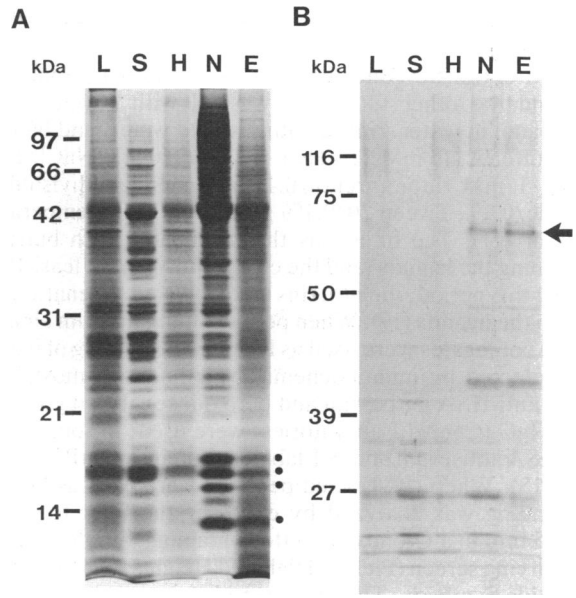

FIG. 3. Intracellular distribution of the 67-kDa nuclear localization sequence-binding protein. (*A*) Coomassie blue-stained 19% gel shows the protein profiles of different subcellular fractions. Lanes: L, low-density membrane fraction; S, soluble fraction; H, high-density membrane fraction; N, purified nuclei; and E, nuclear envelopes. Histone proteins are marked by dots. (*B*) Ligand blot of a $NaDodSO_4$/10.5% polyacrylamide gel containing subcellular fractions probed with wild-type H2B peptide-HSA conjugate and detected with anti-HSA antibody and peroxidase-labeled secondary antibody. The arrow shows p67.

Wild-type H2B peptide-HSA conjugates were used as ligands to probe the nitrocellulose blots of the subcellular fractions. p67 was only present in nuclei or nuclear envelope fractions (Fig. 3*B*). A Coomassie-stained gel of the subcellular fractions is shown in Fig. 3*A*. In contrast to our results, other nuclear localization sequence-binding proteins found in rat liver were reported in both the cytoplasmic and the nuclear envelope fraction. A pure cytosolic fraction was made from yeast cells to verify our negative results with the soluble fraction from the nuclear preparation. In both cases we observed no 67-kDa protein (data not shown for purified cytosol).

Association of p67 with the Nuclear Envelope. To determine the degree of association of p67 with the nucleus, we extracted nuclear envelopes with a series of different reagents (Fig. 4). Yeast nuclei were first treated with DNase I and washed with 0.1 M salt to obtain nuclear envelopes and a DNase I supernatant. The envelopes were then extracted with 0.25 M KCl, 1% Triton X-100, 2% Triton X-100/2 M KCl, or 8 M urea/4 mM EDTA. When blots of the various extractions were probed with wild-type and mutant histone H2B (shown in Fig. 4*A*) or SV40 T-antigen (data, identical to data with H2B, are not shown) peptide-HSA conjugates, the following results were obtained. p67 partitioned solely with the nuclear envelopes and was not present in the DNase I supernatant. This suggests that the protein is not removed with soluble DNA binding proteins. p67 was not extracted with 1% Triton X-100, indicating that it is not an integral membrane protein in the outer nuclear membrane. p67 was only partially extractable with 8 M urea or 2% Triton X-100/2 M KCl (Fig. 4*A*). If p67 were peripherally attached to the nuclear envelope by electrostatic interactions, we would expect it to be extracted with salt. Our data suggest a firm attachment to the nuclear envelope, suggestive of an attachment to the nuclear scaffold or to pore complexes. However,

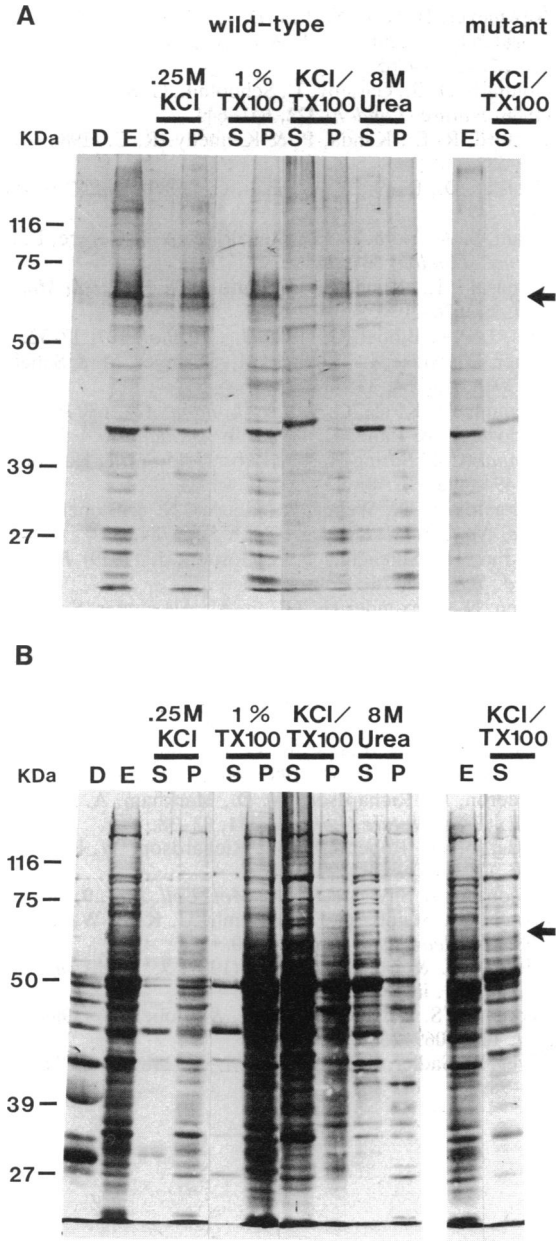

FIG. 4. Subfractionation of purified yeast nuclei and nuclear envelopes. Purified nuclei were digested with DNase I and centrifuged to generate a supernatant (lane D) and nuclear envelope pellet (lanes E). Nuclear envelopes were then extracted with 0.25 M KCl, 1% Triton X-100, 2 M KCl/2% Triton X-100, or 8 M urea (as shown) and centrifuged to give supernatant (lanes S) and pellet (lanes P) fractions. (*A*) Ligand blot of subnuclear fractions probed with wild-type or mutant H2B peptide-HSA conjugate and detected with anti-HSA antibody and peroxidase-labeled secondary antibody. (*B*) Another nitrocellulose filter transferred from the same $NaDodSO_4$ gel as *A* and stained with india ink to visualize the proteins in the various subnuclear fractions. The arrow indicates the position of p67.

without antibodies we cannot assign a specific location to p67. The specificity of binding of the wild-type and mutant peptides to p67 can be reconstituted from the trichloroacetic acid-precipitable fractions. (Fig. 4*A*: see mutant supernatant fraction of KCl/Triton X-100 and compare with the KCl/Triton X-100 supernatant of wild type.)

DISCUSSION

Ligand blotting with free or HSA-conjugated synthetic peptides containing the nuclear localization sequence of histone H2B or SV40 T-antigen allowed the identification of a nuclear envelope protein in yeast that recognized these nuclear import sequences. Two criteria were used to test the specificity of the interaction between the signal-containing peptides and p67. First, the wild-type H2B or SV40 T-antigen signals recognized this protein, but a mutant of the H2B or SV40 T-antigen signal that is incompetent for nuclear transport *in vivo* did not. Second, an excess of free wild-type H2B peptide competed with the wild-type H2B peptide conjugate for binding to p67, but the mutant peptide did not. This result shows a correlation between a functional nuclear localization sequence and the ability to compete for binding to p67. The specificity of the interaction and the inability of mutant nuclear signals to bind to p67 is strong evidence that p67 is involved in nuclear transport. The possibility exists that during the process of ligand blotting, the nuclear envelope proteins do not renature, and the binding that we observe is nonphysiological because of a nonnative or unfolded conformation. However, the combined specificity and binding data with the heterologous wild-type and mutant nuclear localization sequences make this unlikely. Moreover, nuclear localization sequence-binding proteins identified in rat liver nuclear envelopes by Adam *et al.* (11) are masked and unable to efficiently bind the SV40 T-antigen nuclear signal until the proteins are extracted in the nonionic detergent octyl-β-D-glucopyranoside and 0.3 M KCl.

To determine the nature of the association of p67 with the nuclear envelope, a series of subnuclear extractions were carried out. p67 remained associated with the nuclear envelope after DNase I treatment and was not found in the DNase I supernatant. The protein was not extracted from nuclear envelopes with 1% Triton X-100 and only partially extracted with 8 M urea or 2% Triton X-100/2 M KCl. This suggests that p67 is tightly associated with the nuclear envelope. Although subnuclear fractions prepared from rat liver nuclei have been well characterized morphologically, yeast nuclear fractions are less well defined. A paper describing the yeast pore-complex lamina fraction and its apparent similarity to the rat liver nuclei is in press (27), but it is still difficult to assign an exact location for p67—e.g., at the pore complex or in the nuclear envelope membrane—from its biochemical characteristics. However, it is clear from our present data that in yeast the location of p67 is exclusively at the nuclear envelope, and its strong attachment to the envelope is different from that reported for other nuclear localization sequence-binding proteins found in rat liver. A nuclear filament protein of similar molecular weight (66 kDa), lamin B, has recently been identified in yeast (28). This protein has different biochemical characteristics from p67 in that it is quantitatively extracted in 8 M urea, while p67 is only partially extracted. Therefore, p67 is most likely not lamin B.

Nuclear proteins contain sequences that act as a signal to guide them to the nuclear pores (for reviews, see refs. 2 and 3). The actual process of transport across the nuclear envelope has been shown to be composed of two stages, binding and translocation (5–7). The initial recognition of the nuclear signal could occur in alternative ways. A recognition protein could bind nuclear proteins in the cytoplasm and direct them to the nuclear pore complex. Or, the recognition of the nuclear signal could be mediated by a nuclear envelope protein. Our results suggest that recognition of the nuclear localization sequence takes place at the nuclear envelope. In a mammalian system, however, proteins that interact with nuclear localization sequences have been identified recently by two different laboratories; these proteins are both cytoplasmic and/or peripherally associated with the nuclear envelope (11, 12). Their results suggest a cytoplasmic carrier may direct nuclear proteins to the nuclear envelope. Alternatively, the presence of these proteins in the rat liver cytoplasmic fraction could be due to dissociation from the

nuclei during the cell fractionation. However, experiments testing this possibility appeared negative (11). Another possibility is a species difference, although yeast and mammalian proteins function interchangeably for many cellular processes. The differences in rat liver and yeast can only be resolved when the putative nuclear localization sequence-binding proteins are purified, antibodies are made, and the proteins are assayed *in vivo* or *in vitro* for their functional role in the transport of proteins to the nucleus.

Note Added in Proof. While our manuscript was in press, a paper by Silver *et al.* (29) was published describing two proteins of molecular masses 70 kDa and 59 kDa in yeast nuclei that recognize nuclear localization signals. The 70-kDa protein may be identical to p67, whereas we did not observe a 59-kDa protein. The 70-kDa protein and the 67-kDa protein show different biochemical properties—e.g., the 70-kDa protein is totally extracted in 0.5 M NaCl, whereas p67 is only partially extracted under similar conditions. Either the proteins are different or the results could reflect a difference in the methods used to isolate yeast nuclei.

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