Yeast Proteins that Recognize Nuclear Localization Sequences

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Abstract. A variety of peptides can mediate the localization of proteins to the nucleus. We have identified yeast proteins of 70 and 59 kD that bind to nuclear localization peptides of SV-40 T antigen, *Xenopus* nucleoplasmin, and the yeast proteins Gal4 and histone H2B. These proteins are assayed by the binding of peptide–albumin conjugates to proteins immobilized on nitrocellulose filters. These binding proteins fractionate with nuclei and are extractable with salt but not detergent. Radiolabeled peptide–albumin conjugates also bind to isolated nuclei; the binding is saturable and can be extracted with salt. Different nuclear localization peptides compete with each other, implying that a single class of proteins is responsible for their recognition. The 70- and 59-kD proteins have the properties expected for a receptor that would act to direct proteins to the nucleus.

A distinct set of proteins is localized to the nucleus. By one model, transport of proteins from the cytoplasm into the nucleus is triggered by specific interaction of a short amino acid sequence (termed a nuclear localization sequence [NLS][1]) within the transported protein and a receptor, perhaps at the nuclear pore. This model is supported by the existence of discrete nuclear localization sequences within nuclear proteins (Dingwall et al., 1982; Kalderon et al., 1984*a*; Lanford and Butel, 1984; Silver et al., 1984; Hall et al., 1984). These sequences are necessary for specific transport across the nuclear envelope and are sufficient to cause nonnuclear proteins to enter the nucleus.

Recent evidence argues for the existence of an apparatus that would recognize nuclear localization sequences and transport proteins into the nucleus. (*a*) Uptake of proteins into *Xenopus* oocyte nuclei is saturable (Goldfarb et al., 1986); (*b*) Depletion of ATP blocks nuclear protein accumulation both in vivo (Newmeyer et al., 1986*a*) and in vitro (Markland et al., 1987; Newmeyer and Forbes, 1988), consistent with specific transport requiring energy. (*c*) Nuclear protein uptake can be separated into at least two steps: binding at the nuclear envelope followed by ATP-dependent translocation through the pore (Richardson et al., 1988; Newmeyer and Frobes, 1988).

Genetically or chemically conjugated peptides (derived from nuclear proteins) direct nonnuclear proteins to the nucleus (for example, see Kalderon et al., 1984*b*; Silver et al., 1984; Lanford et al., 1986). These peptides contain many basic amino acids, but otherwise have little sequence similarity. One of the best characterized nuclear localization sequences is PKKKRKV, found in SV-40 T antigen between amino acids 126 and 132 (Kalderon et al., 1984*b*; Roberts et al., 1987). We have previously shown that the SV-40 T antigen NLS functions in the yeast, *Saccharomyces cerevisiae*, to direct normally cytoplasmic proteins to the nucleus (Nelson and Silver, 1989). Moreover, a single amino acid change in the SV-40 T antigen NLS reduces its function in animal cells (Kalderon et al., 1984*a*; Lanford and Butel, 1984), as well as in yeast (Nelson and Silver, 1989). From these results, we conclude that the recognition of nuclear localization sequences in yeast is similar to that of mammalian cells.

Other, similarly defined nuclear localization sequences are found in Gal4 (Silver et al., 1984; Nelson and Silver, 1989), *Xenopus* nucleoplasmin (Dingwall et al., 1988), and yeast histone H2B (Moreland et al., 1987). Each of these sequences has been defined as important for nuclear protein localization by a variety of experiments; gene fusions between the SV-40, nucleoplasmin, Gal4, H2B nuclear localization sequences and nonnuclear proteins such as β-galactosidase encode proteins that are localized to the nucleus (Kalderon et al., 1984*a*; Dingwall et al., 1988; Silver et al., 1984; Moreland et al., 1987). In addition, the SV-40 T antigen NLS can be chemically linked to nonnuclear proteins and these hybrids are efficiently nuclear localized when introduced into animal cells (Lanford et al., 1986).

The diversity of sequences that can direct proteins to the nucleus (reviewed in Goodson and Silver, 1989) suggests that multiple receptor proteins might recognize different classes of localization sequences. Alternatively, a single receptor could interact with a diverse set of sequences. We now report the identification of yeast nuclear proteins that specifically bind the SV-40 T antigen NLS, as well as nuclear localization sequences derived from *Xenopus* nuclear protein and two yeast nuclear proteins.

Materials and Methods

Conjugation and Labeling of Peptides

Peptides corresponding to nuclear localization sequences were chemically conjugated to human serum albumin (HSA) and used as probes for nuclear import and binding to nuclear proteins. All peptides were synthesized by a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City,

1. *Abbreviations used in this paper*: HSA, human serum albumin; NLS, nuclear localization sequence.

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CA). Peptides were cleaved with trifluoromethane sulfonic acid, lyophilized, chromatographed on a P4 polyacrylamide resin column in 10% acetic acid followed by a C18 column eluted with a linear gradient of 0.01% trifluoroacetic acid to 70% acetonitrile/0.003% trifluoroacetic acid. Purified peptides were conjugated to HSA (Calbiochem-Behring Corp., San Diego, CA) with the heterobifunctional cross-linker *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, IL) as previously described (Goldfarb et al., 1986). The peptide-HSA conjugate was separated from free peptide by column chromatography with Sephadex G25 in 0.1 M NH_4HCO_3, dialyzed into 10 mM Na-phosphate, pH 7.4, and concentrated with a Centricon microconcentrator (Amicon Corp., Danvers, MA). The degree of conjugation was confirmed by gel electrophoresis on an 8% polyacrylamide gel (Laemmli, 1970).

To test binding to nuclear proteins, peptide–HSA conjugates were radiolabeled. Typically, 250 μg of peptide–HSA conjugate or 9 μg of $Gal4_{(1-147)}$ (purified from an overproducing strain of *Escherichia coli*; Lin et al., 1988) was incubated with Iodo-beads (Pierce Chemical Co.) containing 800 μCi $Na^{125}I$ (Amersham Corp., Arlington Heights, IL) for 12 min at room temperature. The radiolabeled protein was separated from the free iodine by column chromatography with Sephadex G10 in 50 mM Na-phosphate, pH 7.4. This material was used directly for binding to protein blots and to nuclei.

Fluorescently tagged SV-40–HSA was used to examine nuclear protein localization directly. Fluorescent SV-40–HSA and HSA were prepared as follows: 600 μg of protein in 0.1 M sodium carbonate, pH 9/50 mM NaCl was incubated with 200 μg TRITC (Molecular Probes Inc., Junction City, OR) prepared by dilution of 10 mg/ml concentrated stock in dimethylsulfoxide) for 12 h at room temperature. The TRITC-protein was purified by column chromatography with Sephadex G10 in 50 mM Tris-Cl, pH 7.5/50 mM NaCl followed by dialysis with 10 mM Na-phosphate, pH 7.4.

Preparation of Yeast Nuclei

To prepare yeast nuclei, a cell lysate was fractionated on a gradient. The pellet contained the nuclei, while the supernatant contained cytoplasm and other organelles as determined microscopically and biochemically for the presence of cytoplasmic and mitochondrial enzymes. Nuclei were prepared from strains ABYS1 (*pral prbl prcl cpsl ade*) or W303 (*MATa leu2 ura3 trpl ade2 his3 cytl::HIS3*) as described by Allen and Douglas (1989). In brief, cells (2×10^7 cells/ml) were converted to spheroplasts by treatment with zymolyase 60,000 (ICN Radiochemicals, Irvine, CA) and glusulase (Dupont Co., Wilmington, DE), pelleted, and resuspended in lysis buffer (18% Ficoll 400/20 mM Pipes, pH 6.8/0.5 mM $MgCl_2$/1 mM PMSF/1 μg/ml leupeptin, pepstatin, and L-1-Tosylamide-2-phenyl-ethyl chloromethyl ketone [TPCK]). After vortexing at top speed for 3 min, the resulting cell lysate was applied to a glycerol/Ficoll gradient (40, 30, and 20% glycerol cushions in 8% Ficoll 400/20 mM Pipes, pH 6.5/1 mM $MgCl_2$) and centrifuged 23,000 *g* at 4°C. The resulting nuclear pellet was washed twice and resuspended at a concentration of $\sim 1 \times 10^7$ or 1×10^8 nuclei/ml in lysis buffer. Typically, ∼20% of the nuclei were recovered in the purification. The nuclei-depleted fraction (Fig. 4 *A*) corresponds to the supernatant from the gradient and the cytoplasmic fraction was prepared by centrifugation of the total cell lysate for 20 min at 23,000 *g*.

In Vitro Nuclear Localization

To assay nuclear association, the uptake of TRITC-labeled SV-40–HSA by crude or purified nuclei was examined by fluorescence microscopy. Semipermeable yeast cells from yeast strain ABYS1 were prepared by conversion of cells to spheroplasts with zymolyase. The spheroplasts were pelleted and resuspended in 25 mM Hepes, pH 7/150 mM KCl/2 mM $MgCl_2$/2 mM DTT/250 mM sorbitol and permeabilized by 10-fold dilution into the same buffer lacking sorbitol and containing TRITC–SV-40–HSA or TRITC–HSA (both at 200 μg/ml). After 10 min at room temperature DAPI was added (50 ng/ml final concentration), the cells placed on a glass slide and viewed by fluorescence microscopy. For experiments with isolated nuclei, nuclei prepared as described above were resuspended in 18% Ficoll in buffer A (50 mM Tris-Cl, pH 7.2/25 mM KCl/2.5 mM $MgCl_2$/3 mM $CaCl_2$/1 mg/ml leupeptin, pepstatin, and TPCK) at $0.5\text{-}1.0 \times 10^7$ nuclei/ml, mixed with TRITC–SV-40–HSA or TRITC–HSA (200 μg/ml) at a ratio of four parts nuclei to one part TRITC-protein. ATP (1 mM final concentration) was added, the reaction incubated for 10 min at 30°C. Nuclei were viewed directly or were collected by centrifugation for 10 min at 14,000 *g* at 4°C, washed once, and resuspended in 18% Ficoll/buffer A and then viewed by fluorescence microscopy. All experiments used a Zeiss Axioscope equipped for fluorescence microscopy with Nomarski optics.

Binding of Peptide–HSA Conjugates to Nuclei

The association of peptide–HSA with nuclei was examined as follows. Nuclei (1×10^8 nuclei/ml in lysis buffer) were mixed with the ^{125}I-peptide–HSA conjugate in 5% BSA/buffer A/18% Ficoll, incubated for 30 min at 4°C, collected by centrifugation at 15,000 *g* for 10 min, resuspended in 5% BSA/18% Ficoll/buffer A, transferred to a fresh tube, centrifuged for 10 min at 15,000 *g*, and the amount of bound and unbound protein determined by counting samples in the γ counter.

Protein Blots

Interactions between nuclear localization peptides and yeast proteins were detected by a Western blot type assay (Burnette, 1981). Proteins were electrophoresed through an SDS polyacrylamide gel, transferred to nitrocellulose by electroblotting, and the resulting filter probed with ^{125}I-peptide–HSA. Samples to be analyzed for NLS binding activity were solubilized in gel sample buffer, heated at 90°C for 5 min, and electrophoresed on a 10% SDS polyacrylamide gel (Laemmli, 1970). After electrophoresis, proteins were transferred to nitrocellulose with a transfer unit (model TE52; Hoefer Scientific Instruments, San Francisco, CA) in 24 mM Tris/190 mM glycine at 4°C for 12 h. After transfer, the nitrocellulose blot was blocked with 5% BSA in buffer A for 2 h at room temperature and for 1 h at 37°C, incubated with ^{125}I-protein conjugate in 5% BSA/buffer A for 12 h at 4°C, washed in buffer A at room temperature, and exposed to x-ray film with an intensifying screen at −70°C.

Results

To generate biochemical probes for nuclear localization in vitro, different nuclear localization peptides and either the radioactive label ^{125}I or the fluorescent label TRITC were chemically linked to HSA. Radioactive labeling of the peptide–HSA conjugate rather than the peptide alone amplifies the signal associated with each substrate molecule. In these experiments, peptide conjugates (Fig. 1) are referred to by their source protein: SV-40–HSA refers to the SV-40 large T antigen nuclear localization peptide, SV-40*–HSA to a mutant form of the SV-40 peptide with the lysine converted to threonine, NP–HSA to a peptide derived from the *Xenopus* nucleoplasmin, and H2B–HSA to a peptide from the yeast histone H2B. These NLS–HSA conjugates were used in binding assays to detect the presence of proteins that function in nuclear import.

Nuclear Localization Peptide Conjugates Bind to Isolated Yeast Nuclei In Vitro

The SV-40 T antigen nuclear localization sequence is recognized by yeast nuclei in vitro. A peptide with the sequence CTPPKKKRKV (Fig. 1), was chemically cross-linked to HSA (Goldfarb et al., 1986). Most of the resulting population of peptide–protein conjugates had 15–20 peptides conjugated per molecule of albumin as determined by relative

SV40 NH_2- C - T - P - P - K - K - K - R - K - V - COOH

SV40* NH_2- C - T - P - P - K - T - K - R - K - V - COOH

NP NH_2- C - A - V - K - R - P - A - A - T - K - K - A - G - Q - A - K - K - K - COOH

H2B NH_2- C - P - P - G - K - K - R - S - K - A - COOH

Figure 1. Peptides used in these studies. Peptides corresponding to nuclear localization sequences from SV-40 large T antigen (*SV40*), *Xenopus* nucleoplasmin (*NP*), *S. cerevisiae* histone H2B (*H2B*), and a mutated NLS (*SV40**) were synthesized and conjugated to human serum albumin as described in Materials and Methods.

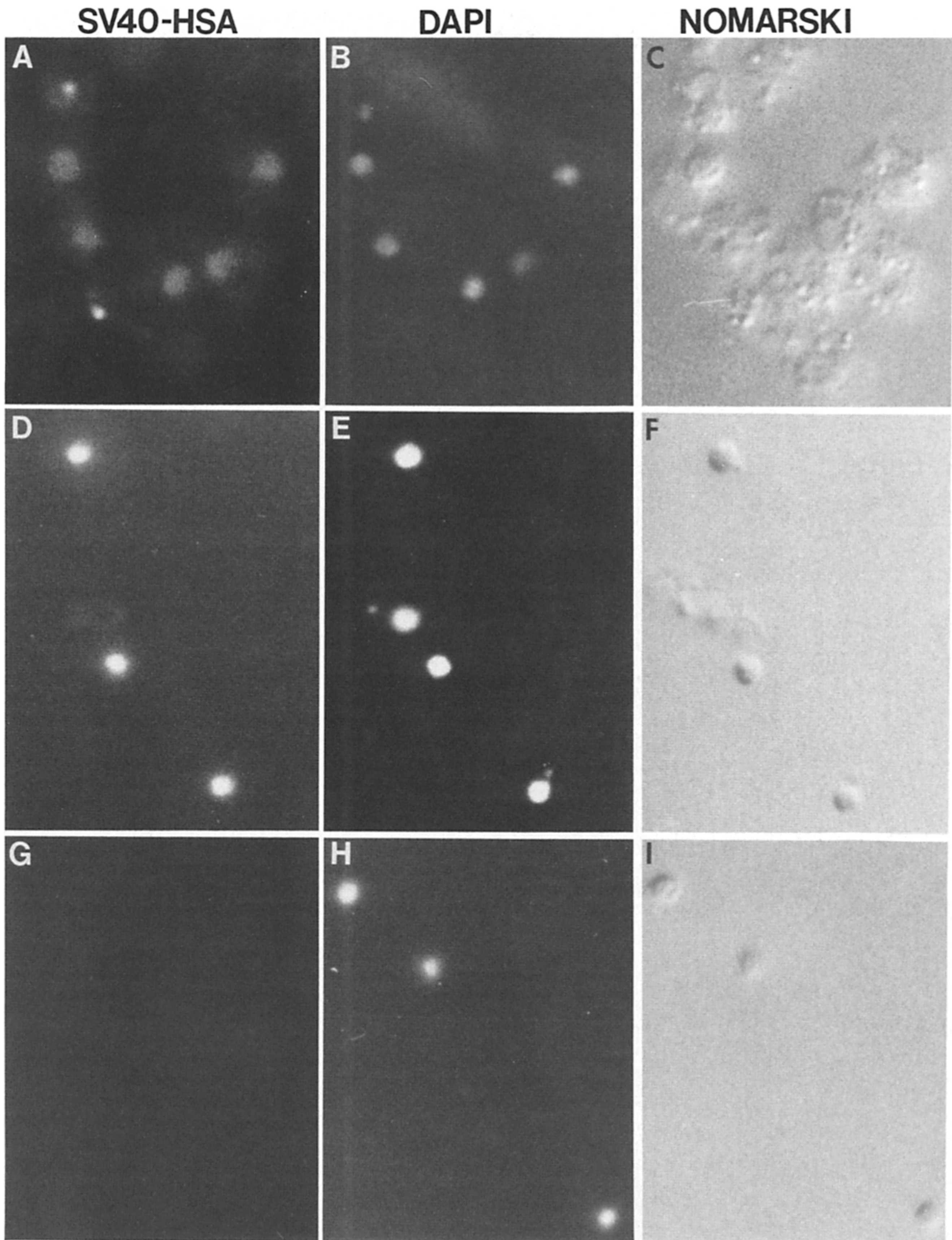

Figure 2. Association of TRITC SV-40-HSA with yeast nuclei in vitro. Semipermeable yeast cells from yeast strain ABYS1 were prepared and mixed with TRITC SV-40-HSA as described in Materials and Methods (*A–C*). Nuclei from strain (W303) were prepared as described in Materials and Methods and mixed with TRITC SV-40-HSA (*D–F*) or TRITC SV-40*-HSA (*G–I*) with ATP (2 mM) in buffer A (50 mM Tris-Cl, pH 7.2/25 mM KCl/2.5 mM $MgCl_2$/3 mM $CaCl_2$/1 mg/ml leupeptin, pepstatin, and TPCK). *A* and *D*, TRITC SV-40-HSA; *G*, TRITC SV-40*-HSA; *B*, *E*, and *H*, DAPI to visualize nuclei; *C*, *F*, and *I*, Nomarski optics to visualize cells and nuclei. SV-40-HSA and SV-40*-HSA were conjugated with the same amount of TRITC.

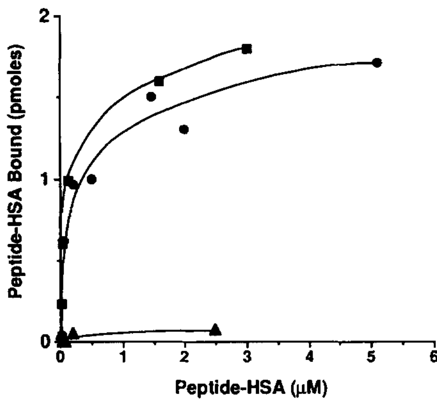

Figure 3. Binding of ^{125}I peptide albumin conjugates to isolated yeast nuclei. Binding assays were performed by incubation of nuclei with ^{125}I-labeled SV-40-HSA (3 × 10^3 cpm/μg, ■), NP-HSA (9 × 10^3 cpm/μg, ●), and SV-40*-HSA (4 × 10^3 cpm/mg, ▲) for 30 min at 4°C as described in Materials and Methods.

mobility on a SDS polyacrylamide gel. The SV-40-HSA was fluorescently tagged with TRITC (Newmeyer et al., 1986). Upon incubation with osmotically shocked yeast cells that are permeable to macromolecules (Ruhola et al., 1988), the fluorescent SV-40-HSA was associated with some nuclei as judged by fluorescence microscopy (Fig. 2, *A-C*). Purified yeast nuclei also bound the TRITC SV-40-HSA (Fig. 2, *D-F*). Only nuclei that appeared intact by Normarski bound sufficient TRITC SV-40-HSA to be visualized. Nuclei that appeared lysed, although they still stained positive with DAPI, did not show any association of the TRITC-SV-40-HSA. TRITC-SV-40*-HSA showed reduced association with isolated nuclei (Fig. 2, *G-I*) and TRITC-HSA did not associate with isolated yeast nuclei under the same conditions (data not shown).

To quantitate the amount of SV-40-HSA nuclear binding, we measured the association of ^{125}I-SV-40-HSA with isolated nuclei (Fig. 3). This was saturable and the SV-40*-HSA had 100-fold reduced affinity. NP-HSA also bound to isolated nuclei and was saturable. The extent of binding of the SV-40- and the NP-HSA was about equal. Together, the above results indicate that isolated yeast nuclei are capable of recognizing nuclear localization peptides.

Binding of SV-40 Nuclear Localization Peptide to Immobilized Nuclear Proteins

Two major proteins of 70 and 59 kD were recognized by the SV-40 NLS, as judged by the gel binding assay (see Materials and Methods). Yeast nuclei were resuspended in gel sample buffer (Laemmli, 1970), electrophoresed through a 10% SDS polyacrylamide gel, and transferred to nitrocellulose by electroblotting. After blocking in BSA, the nitrocellulose filter was incubated with ^{125}I-labeled SV-40-HSA and exposed to film. Two major bands were observed corresponding to proteins of molecular weights 70 and 59 kD (Fig. 4 *A*, lane *2*). Minor bands corresponding to 95 and 140 kD were also occasionally observed. The 70 and 59 kD bands did not correspond to abundant nuclear proteins as judged by Coomassie staining of the extract (Fig. 4 *B*, lane *3*). Cell lysates depleted of nuclei (Fig. 4 *A*, lane *4*), as well as a similarly treated protein extract from *E. coli* showed no binding of the ^{125}I-SV-40-HSA (Fig. 4 *A*, lane *1*). All of the binding activity was recovered with the yeast nuclear pellet and none was found in the soluble (cytoplasmic) fraction from lysed cells (Fig. 4 *A*, lane *5*).

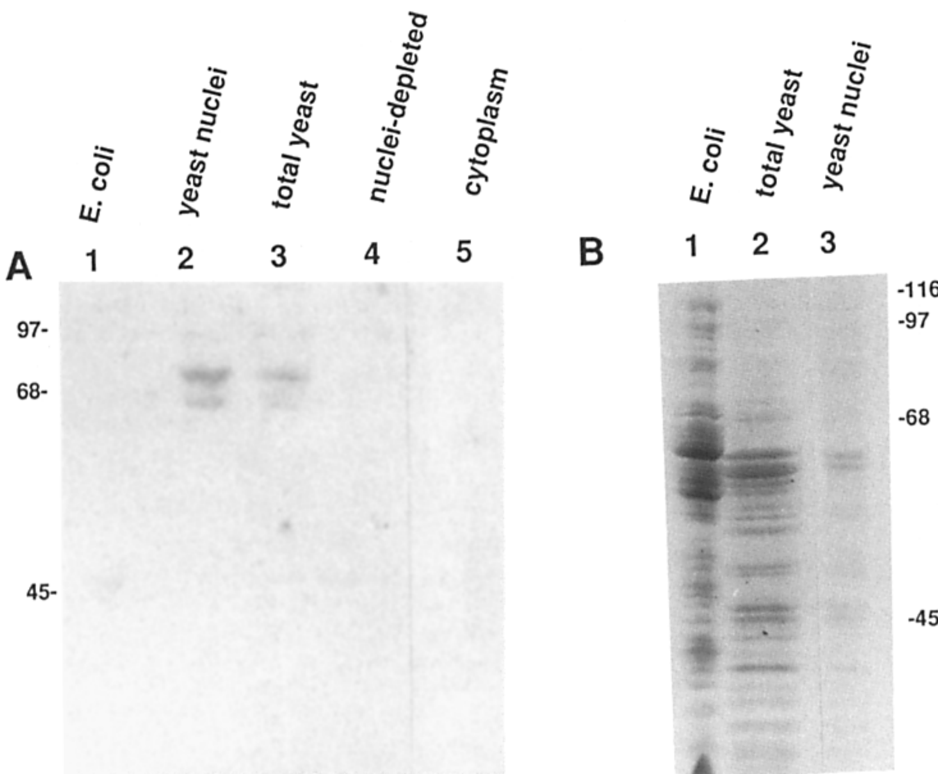

Figure 4. Identification of SV-40 NLS binding proteins by binding to proteins immobilized on nitrocellulose. (*A*) Samples were resuspended in SDS gel sample buffer (Laemmli, 1970), electrophoresed on a 10% polyacrylamide gel, the proteins electroblotted to nitrocellulose, and the blot treated as described in Materials and Methods. After blocking with 5% BSA in buffer A, the blot was incubated with ^{125}I-SV-40-HSA (6 μCi/μg). Lane *1*, *E. coli* (JM101) extract; lane *2*, yeast nuclei; lane *3*, total yeast extract; lane *4*, yeast extract depleted of nuclei; lane *5*, yeast cytoplasmic fraction. Equal cell equivalents were loaded in lanes *3-5* and two cell equivalents in lane *2*. (*B*) Coomassie brilliant blue-stained 10% polyacrylamide gel of lane *1*, *E. coli* extract; lane *2*, total yeast extract; lane *3*, yeast nuclei. Molecular weight standards were myosin (200 kD), β-galactosidase (116 kD), phosphorylase (97 kD), bovine serum albumin (68 kD), and chicken egg albumin (45 kD).

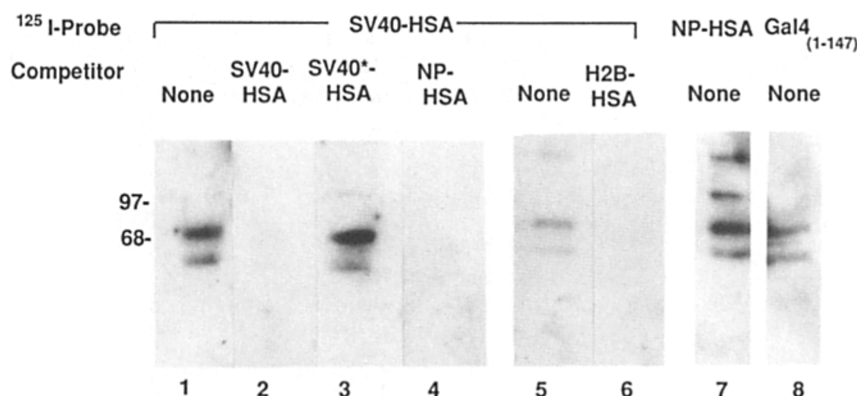

Figure 5. NLS binding proteins recognize different nuclear localization sequences. Equal amounts of yeast nuclei (from ABYS1) were electrophoresed on a 10% polyacrylamide gel, blotted to nitrocellulose, treated as described in Materials and Methods, and the filter probed with: lane *1*, ^{125}I-SV-40-HSA; lane *2*, ^{125}I-SV-40-HSA and 100-fold chemical excess of unlabeled SV-40-HSA; lane *3*, ^{125}I-SV-40-HSA and 100-fold chemical excess of SV-40*-HSA; lane *4*, ^{125}I-SV-40-HSA and 100-fold chemical excess of NP-HSA; lane *5*, ^{125}I-SV-40-HSA; lane *6*, ^{125}I-SV-40-HSA and 100-fold chemical excess of H2B-HSA; lane *7*, ^{125}I-NP-HSA; lane *8*, $Gal4_{(1-147)}$. For lanes *1-7*, the concentration of ^{125}I-peptide-HSA was 2.5 μg/ml (6 μCi/μg) and of unlabeled peptide-HSA was 250 μg/ml. For the experiment in lane *8*, ^{125}I-Gal4 at 0.08 μg/ml (2 μCi/μg) was used.

Binding to the 70- and 59-kD proteins is specific for the wild-type SV-40 peptide. When the blot containing the nuclear extract was incubated with both ^{125}I-SV-40-HSA and a 100-fold chemical excess of unlabeled SV-40-HSA, the radiolabeled bands were no longer visible (Fig. 5, lane *2*). However, when the blot was incubated with ^{125}I-SV-40-HSA and 100-fold chemical excess of unlabeled HSA or SV-40*-HSA (Fig. 1), no diminution in the binding was observed (Fig. 5, lane *3*). Moreover, when a blot containing a nuclear extract was incubated directly with ^{125}I-SV-40*-HSA, no binding to any proteins was observed (data not shown).

Binding of Other Peptides to Immobilized Nuclear Proteins

Different nuclear localization peptides compete with each other for binding to the 70 and 59 kD proteins. Binding of ^{125}I-SV-40-HSA was inhibited by the presence of 100-fold chemical excess of either NP-HSA or H2B-HSA (Fig. 5, lanes *4-6*). When ^{125}I-NP-HSA conjugate was incubated with blots containing yeast nuclear extracts, binding to the 70- and 59-kD proteins was observed directly (Fig. 5, lane *7*). Additional binding of NP-HSA to the 95- and 140-kD proteins was also often observed. When a radiolabeled protein fragment containing Gal4 amino acids 1-147 (termed $Gal4_{1-147}$) was used to probe a blot containing yeast nuclear proteins, two bands of 70 and 59 kD were observed (Fig. 5, lane *8*). The Gal4 binding could also be competed by addition of excess of unlabeled SV-40-HSA, NP-HSA, and H2B-HSA (data not shown). These results suggest that the same yeast proteins are capable of binding to distinct nuclear localization sequences. We refer to these proteins as NLS binding proteins.

Characterization of NLS Binding Proteins

The NLS binding activity, assayed by binding to proteins immobilized on nitrocellulose, can be partially solubilized by high salt but not by detergent. When nuclei were incubated with Triton X-100 and collected by centrifugation, all of the binding activity remained associated with nuclei (Fig. 6, lane *10*). When nuclei were treated with 0.5 M or 1 M NaCl (Fig. 6, lanes *3* and *4*), 50-90% of the activity was released into the supernatant (Fig. 6, lanes *7* and *8*). Treatment with 2.5 mM $MgCl_2$ and 3 mM $CaCl_2$ had no effect (Fig. 6, lanes *1*, *2*, *5*, and *6*). This binding activity was completely eliminated by protease treatment of nuclei (Fig. 6, lanes *11* and *12*).

Discussion

The existence of nuclear localization sequences within proteins suggests that specific receptors may recognize proteins destined for the nucleus. Isolated nuclei from *Saccharomyces cerevisiae* bind to nuclear localization peptides conjugated to albumin (Figs. 1 and 2). This binding has the hallmarks of a receptor-ligand interaction: the binding is saturable and specific.

From isolated nuclei, we identified proteins of 70 and 59 kD that also bind to nuclear localization peptides. Binding was assayed using a Western blot-type assay. The properties of these proteins suggest that they are receptors for nuclear proteins. Binding to the 70- and 59-kD proteins is competitive, and mutant peptides do not bind or compete. Different peptide conjugates also cross-compete for binding to the 70- and 59-kD proteins, suggesting that they bind to the same sites. Finally, the binding is not a consequence of high peptide concentration on the conjugates, because a fragment of the yeast DNA binding protein, Gal4, binds to the same proteins. (This Gal4 fragment contains sufficient information for nuclear import [Silver et al., 1984; Nelson and Silver, 1989]).

Peptides derived from the nonyeast proteins, *Xenopus* nucleoplasmin and SV-40 T antigen, recognize the same proteins as those recognized by yeast nuclear localization sequences. This is based on cross-competition between NP-HSA, SV-40-HSA, and H2B-HSA, as well as direct binding to proteins of the same size. These findings are consistent with our observations that the SV-40 and nucleoplasmin nuclear localization sequences are recognized correctly by yeast in vivo (Nelson and Silver, 1989; Sadler, I., and P. A. Silver, manuscript in preparation).

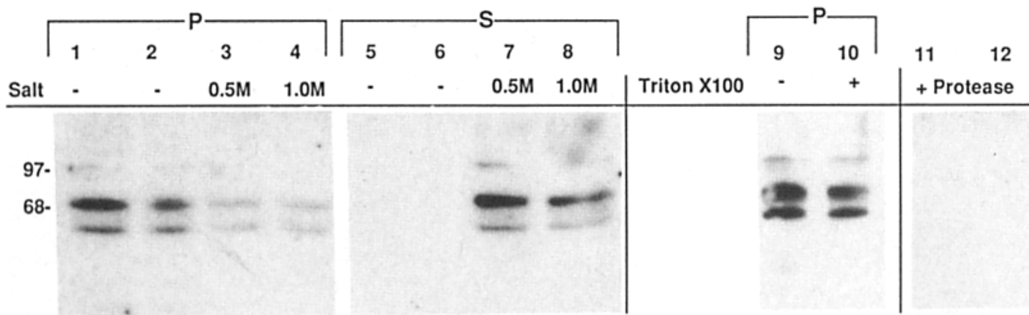

Figure 6. Characterization of NLS binding activity. Yeast nuclei (from ABYS1) were resuspended in buffer A and treated with either NaCl (lanes *3*, *4*, *7*, and *8*), Triton X-100 (lanes *9* and *10*), or protease (lanes *11* and *12*), collected by centrifugation, the nuclear pellet (*P*) and postnuclear supernatant (*S*) were electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose, and the filter probed with ^{125}I-SV-40–HSA (0.04 mCi/ml). Nuclear pellets after 30 min at 4°C (lane *1* and *9*); 30 min at 37°C (lane *2*); 0.5 M NaCl, 30 min 4°C (lane *3*); 1 M NaCl 30 min at 4°C (lane *4*); 30 min at 4°C in 2% Triton X-100 (lane *10*); plus trypsin (0.5 mg/ml), 30 min at 37°C (lane *11*); plus pronase (0.5 mg/ml), 30 min at 37°C (lane *12*). Postnuclear supernatants after 30 min at 4°C (lane *5*); 30 min at 37°C (lane *6*); 0.5 M NaCl 30 min at 4°C (lane *7*); and 1 M NaCl 30 min at 4°C (lane *8*). Equal amounts of nuclei were loaded in lanes *1–8* and *11* and *12*, and twice the amount of nuclei in lanes *9* and *10*.

The biochemical behavior of the proteins suggests that they are associated with nuclei via protein–protein interactions. Binding activity can be extracted from nuclei with salt, but not detergent. Upon treatment with salt, the 70- and 59-kD binding proteins are released (Fig. 6). The amount extracted varies and may reflect populations of protein associated in different ways with the nuclei. Attempts to restore full binding activity by adding back salt-extracted proteins have, thus far, not been successful.

Others (Adam et al., 1989) have identified proteins from rat liver of ~70 and 59 kD that appear to be involved in the localization of proteins to the nucleus. Unlike the yeast proteins described here, the rat liver proteins are found distributed between the nucleus and the cytoplasm. However, the nuclei were treated with higher salt before assaying the NLS binding activity. Our assay also detected proteins of similar size in rat liver nuclei (Seibel, J., and P. A. Silver, unpublished observations). One possibility is that the NLS-binding proteins recognize nuclear-destined proteins in the cytoplasm as they emerge from the ribosome. After binding, the proteins are transported to the nuclear pore where they are subsequently imported. The differences in nuclear association of the binding proteins may represent proteins at different stages of import.

Yoneda et al. (1988) reasoned that since all known nuclear localization sequences are basic, a receptor for these proteins may be acidic. They developed antibodies against the peptide DDDED and found that these antibodies reacted with rat liver nuclear proteins of 69 and 59 kD. In addition, when the antibodies are microinjected into HEL cells, nuclear import is inhibited. Immunofluorescence with these antibodies revealed punctate staining of rat fibroblast nuclei, suggesting association of the antigen with the nuclear pores.

A high content of basic amino acids is not sufficient to confer nuclear localization activity upon the SV-40 peptide. Removal of positive charge by conversion of lysine 128 to threonine eliminates SV-40 T antigen nuclear localization (Lanford and Butel, 1984; Kalderon et al., 1984*a*). However, replacement of the neighboring proline by lysine did not restore import (Colledge et al., 1986). This situation is reminiscent of that of other localization sequences: signal sequences for secretion are generally hydrophobic, and those for mitochondrial localization are a combination of basic and hydrophobic residues. Similarly, transcriptionally activating "acid blobs" are all acidic but have little primary sequence homology (Ma and Ptashne, 1987). In each case, there is a semi-specific interaction; many different ligands are allowed, while other potential ligands are excluded. The results in this paper illustrate that semi-specific interactions can be mediated by a single set of proteins.

The NLS binding proteins retain their binding ability despite heating, SDS gel electrophoresis, and electroblotting. GTP binding proteins (Lapetina and Reep, 1987), DNA binding proteins (Lin and Riggs, 1975; Fletcher et al., 1987; Prywes and Roeder, 1987), and the low density lipoprotein receptor (Daniel et al., 1983) also retain activity after similar treatment. One possible explanation for the retention of activity is that only a small region of the protein is responsible for binding to the NLS. Alternatively, some of the protein may renature as the SDS is removed during electroblotting. The partially denatured, electroblotted protein may then only possess a small fraction of the total binding activity of the native protein. In separate experiments, we have detected NLS binding activity in nuclear extracts bound directly to nitrocellulose by filter binding.

The two major binding proteins, distinguished by their mobility on an SDS gel, may be distinct or may be related. While the smaller protein may be a degradation product, in a protease deficient yeast strain (ABYS1), we still observed both binding species. Chemical modifications may also explain altered mobilities.

Nuclear protein import has been proposed to be an active process catalyzed by recognition of nuclear localization sequences and subsequent ATP-dependent transfer through the pore (Feldherr et al., 1984; Newmeyer and Forbes, 1988). We propose that the proteins identified here are part of a recognition apparatus in the yeast *S. cerevisiae* and may be analogous to proteins identified in mammalian cells (Yoneda et al., 1988; Adam et al., 1989).

We especially thank M. Flocco for peptide synthesis; M. Carey for purified Gal4 protein; and J. Way and M. Bernstein for comments on the manuscript.

This work was supported by National Institutes of Health grant GM36373-01 and in part by a Presidential Young Investigator Award from the National Science Foundation to P. Silver. I. Sadler is supported by Deutscher Akademischer Austauschdienst and Deutsche Forschungsgemeinschaft postdoctoral fellowships.

Received for publication 6 March 1989, and in revised form 2 May 1989.

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