# A Yeast Protein That Binds Nuclear Localization Signals: Purification Localization, and Antibody Inhibition of Binding Activity

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*Abstract.* Short stretches of amino acids, termed nuclear localization sequences (NLS), can mediate assembly of proteins into the nucleus. Proteins from the yeast, *Saccharomyces cerevisiae*, have been identified that specifically recognize nuclear localization peptides (Silver, P., I. Sadler, and M. A. Osborne. 1989. *J. Cell Biol.* 109:983–989). We now further define the role of one of these NLS-binding proteins in nuclear protein localization. The NLS-binding protein of 70-kD molecular mass can be purified from salt extracts of nuclei. Antibodies raised against the NLS-binding protein localized the protein mainly to the nucleus with minor amounts in the cytoplasm. These antibodies also inhibited the association of NLS-protein conjugates with nuclei. Incubation of nuclei with proteases coupled to agarose removed NLS-binding protein activity. Extracts enriched for NLS-binding proteins can be added back to salt or protease-treated nuclei to restore NLS-binding activity. These results suggest that the first step of nuclear protein import can be reconstituted in vitro.

THE nucleus is characterized by its distinct set of proteins. One model for nuclear protein uptake proposes that it is an active process, triggered by specific interaction of a short stretch of amino acids contained within the transported protein (termed nuclear localization sequence [NLS][1]) and one or more "receptors," perhaps located at the nuclear pore complex. Nuclear localization sequences are found in many nuclear proteins (for examples see Kalderon et al., 1984; Hall et al., 1984; and Silver et al., 1984) and are necessary for protein transit across the nuclear envelope via the nuclear pores (Feldherr et al., 1984; Richardson et al., 1988; Newmeyer and Forbes, 1988).

Specific information for nuclear protein localization resides not only within the transported protein as evidenced by the presence of nuclear localization sequences, but also in the transport apparatus. Evidence for receptors that recognize proteins for nuclear import was first provided by Goldfarb et al. (1986), who cross-linked BSA to the SV40 T-antigen NLS and found that the rate of nuclear uptake for the complex was saturable. Further support for receptor-mediated uptake comes from microinjection of nuclear proteins into animal cells (Lanford et al., 1986; Richardson et al., 1988; Breeuwer and Goldfarb, 1990) and in vitro experiments with nuclei from rat liver (Markland et al., 1987), *Xenopus* oocytes (Newmeyer and Forbes, 1988; Newmeyer et al., 1986), and yeast (Kalinich and Douglas, 1989). Nuclei will import SV40 T-antigen and *Xenopus* nucleoplasmin in a manner that is dependent on the NLS, temperature, and ATP. In the absence of ATP, gold particles coated with the SV40 NLS bind at the pore, but are not imported, suggesting that nuclear localization is at least a two-step process: binding followed by translocation through the pore (Richardson et al., 1988; Newmeyer and Forbes, 1988).

Nuclear and cytoplasmic proteins from yeast (Silver et al., 1989; Lee and Melese, 1989) and animal cells (Yoneda et al., 1988; Adam et al., 1989; Yamasaki et al., 1989; Meier and Blobel, 1990) have been identified that specifically interact with nuclear localization sequences from a number of different nuclear proteins. For instance, these proteins bind the wild-type SV40 NLS but not a localization-defective mutant and, thus, have the properties expected for a receptor that would act to direct proteins to the nucleus. However, we do not yet know at which step in protein import these proteins might act. NLS-binding proteins might be components of the nuclear pore complex and associate with proteins prior to their import. Alternatively, they could be transiently bound to the nuclear pore complex and cycle between the cytoplasm and the nucleus.

To define more precisely the role of the NLS-binding proteins from the yeast *Saccharomyces cerevisiae*, we have purified the NLS-binding protein of 70 kD. Results are presented that support the hypothesis that the 70-kD NLS-binding protein is an important component of the nuclear protein import machinery in yeast.

## Materials and Methods

Yeast nuclei were prepared from yeast strain ABYS1 (*pral prbl prcl cps1 ade2*). W303 (*MATa ade2-1 trp1-1 ura3-1 leu2-3, 112 his3-11,15 can1-100*) and tetraploid S288C (*MATα mal gal2*) were used for immunofluorescence. Synthesis, conjugation, and radiolabeling of peptide-albumin conjugates and nuclear proteins were carried out as previously described (Silver et al., 1989). The amino acid content of all peptides was confirmed by sequencing.

1. *Abbreviation used in this paper*: NLS, nuclear localization sequence.

## Affinity Chromatography

Affinity resins were prepared by incubation of peptides corresponding to NLSs of wild-type (CTPPKKKRKV) or mutant (CTPPKTKRKV) SV40 large T antigen as well as *Xenopus* nucleoplasmin (CAVKRPAATKKAGQA-KKK) with Affi-Gel 401 (BioRad Laboratories, Richmond, CA) in 10 mM Tris-HCl, pH 7.5, for 12 h at RT. Unconjugated peptides were removed by centrifugation (5,000 *g*, 5 min). The coupling efficiency for each peptide was 70–80% as determined by UV absorption. Approximately 4 mg peptide were coupled per ml of Affi-Gel. Nucleoplasmin NLS-HSA conjugates were coupled to CNBr-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) as previously described (Kilmartin and Adams, 1984).

To purify NLS-binding proteins, peptide affinity resins were incubated batchwise with yeast extracts containing aprotinin, antipain, chymostatin, leupeptin, and pepstatin A (each at 0.1 μg/ml) for 1 h at RT, and washed with buffer A (50 mM Tris-HCl, pH 7.2, 25 mM KCl, 2.5 mM $MgCl_2$, 3 mM $CaCl_2$, 20% (vol/vol) glycerol) supplemented with different concentrations of NaCl or free NLS-peptide as indicated in the figure legends. Released NLS-binding proteins were analyzed by either the dot blot or gel binding assays.

## Assays for NLS-binding Proteins

For the dot-blot assay, proteins were applied directly to nitrocellulose filters. The filters were then incubated with 5% BSA in buffer A for 1 h at RT to block nonspecific binding sites, followed by incubation for 12 h at 4°C with $^{125}$I-SV40-HSA (0.1 μCi/ml) in buffer A/5% BSA, and finally washed with buffer A for 10 min at RT. The washed filters were exposed to Kodak X-AR5 film with an intensifying screen and dots were excised from the nitrocellulose and the amount of bound radioactivity was determined with a γ-counter (triplicate determinations had deviations no greater than 5%) and used to calculate specific activities. A linear relationship between the amount of protein applied to nitrocellulose and $^{125}$I-SV40-HSA bound was obtained between 0 and 12.5 μg per dot. For the gel binding assay, proteins were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and the filters probed with $^{125}$I-SV40-HSA as previously described (Silver et al., 1989). Specific activities were also calculated by excision of the labeled band corresponding to the 70-kD NLS-binding protein and the amount of radioactivity was determined with a γ-counter.

## Preparation of DEAE Extracts

Yeast cells were lysed with 0.5-mm glass beads in 0.1 M Tris-HCl, pH 8.0, 1 M NaCl, 1 mM PMSF, and pepstatin, leupeptin, and TPCK (each at 0.3 μg/ml) at 4°C in a bead beater (Biospec, Bartlesville, OK) for 10 min in 1-min intervals. Lysis was followed by microscopic examination and was typically >90%. Lysates were centrifuged for 5 min at 2,500 *g*, the resulting supernatant centrifuged for 60 min at 25,000 *g*, followed by centrifugation for 60 min at 150,000 *g*. The final supernatant was dialyzed versus 0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl. The dialyzed material was centrifuged for 15 min, 25,000 *g* and the supernatant applied to DEAE-FF-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) preequilibrated with 0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 1 mM PMSF. Bound protein was eluted by a continuous gradient of 0.1 to 1 M NaCl in 0.1 M Tris-HCl, pH 8.0, 1 mM PMSF. Fractions were analyzed for NLS-binding activity by the dot-blot assay.

## Salt and Protease Treatment of Nuclei

To prepare nuclei, a cell lysate was fractionated on a gradient as previously described (Silver et al., 1989). A cytosolic fraction was prepared from the gradient supernatant by 10-fold dilution in buffer A followed by centrifugation for 30 min at 41,000 *g*. The resulting supernatant was further centrifuged for 1 h at 150,000 *g*.

For salt extraction, ~1 × $10^8$ nuclei were incubated for 30 min on ice in buffer A supplemented with protease inhibitors (antipain, aprotinin, chymostatin, leupeptin, pepstatin all at 0.1 μg/ml) and 0.3 M NaCl. Nuclei were then collected by centrifugation for 2 min at 14,000 *g*, reextracted twice, and washed in buffer A without salt. Control nuclei were treated identically with omission of NaCl in the incubation mixture. Salt-extracted proteins were concentrated with a microconcentrator (cutoff $M_r$ = 10,000; Amicon Corp., Danvers, MA) and dialyzed versus buffer A containing 50 mM NaCl. Extracts stored at −70°C retained their activity for 2–3 wk.

For treatment with proteases, ~1 × $10^7$ nuclei were incubated with trypsin conjugated to agarose (Sigma Chemical Co.) in buffer A (8 U/ml) for 45 min at 4°C. Proteolysis was terminated by addition of 2.5 mM PMSF and 1 mg/ml aprotinin.

## Reconstitution of NLS-dependent Binding to Nuclei

For determination of binding of SV40-HSA, $10^7$ nuclei in 100 μl were incubated with 0.6 μCi/ml $^{125}$I-SV40-HSA (~2 μCi/μg) for 15 min at 4°C in buffer A/2% BSA/protease inhibitors (antipain, aprotinin, chymostatin, leupeptin, pepstatin A all at 0.1 μg/ml), collected by centrifugation for 2 min at 14,000 *g*, and washed by gentle vortexing with buffer A/2% BSA. Association of $^{125}$I-SV40-HSA was determined by counting samples in the γ-counter. To correct for nonspecific binding, samples were treated identically but in the absence of nuclei or with an excess of unlabeled SV40-HSA (which gave the same results). By this method, we estimate that ~1,000–10,000 molecules of SV40-HSA were bound per nucleus.

For reconstitution experiments, salt or trypsin-agarose–treated nuclei were preincubated in buffer A/2% BSA/protease inhibitors (antipain, aprotinin, chymostatin, leupeptin, pepstatin A all at 0.1 μg/ml), then supplemented with salt extracts containing 50 μg of protein or DEAE-purified fractions containing 20 μg protein for 30 min at 4°C. Association of $^{125}$I-SV40-HSA was determined after collection of nuclei by centrifugation for 2 min at 14,000 *g*.

## Preparation of Antibodies

Polyclonal antibodies directed against the 70-kD NLS-binding protein were raised in mice according to Hamatake et al., 1990. Briefly, after affinity chromatography, NLS-binding proteins were electrophoresed in SDS-polyacrylamide gels and transferred to Immobilon-P membranes. Transferred proteins were visualized by staining with amido black. The 70-kD polypeptides were eluted with 0.1 M Tris-HCl, pH 9/4% (wt/vol) SDS and used for immunizations.

Affinity purification of anti-p70 antibodies was carried out with 70 kD NLS-binding protein immobilized on nitrocellulose. Nonspecific binding sites were blocked with 1 mg/ml BSA in PBS (1 h/RT) followed by incubation with antisera diluted 1 to 10 in BSA/PBS (1 mg/ml) for 30 min at RT. Filters were washed several times with PBS and 20 mM sodium phosphate, pH 7.5, 130 mM NaCl. Bound antibodies were released with 0.1 M glycine, pH 2.8/0.5 M NaCl/0.1% BSA. The pH was neutralized by addition of 1 M Tris-HCl, pH 9.5, and antibodies were subsequently dialyzed against buffer A.

Polyclonal antibodies against the NspI peptide CSKPAFSGAK were raised in rabbits after coupling to KLH as described (Silver et al., 1989). Affinity purification of antibodies was carried out with peptide covalently attached to SulfoLink (Pierce Chemical Co., Rockford, IL) according to the suppliers. To determine the effect on NLS-mediated nuclear binding, affinity-purified antibodies were dialyzed against buffer A.

## Immunofluorescence

Immunofluorescence was performed on intact yeast cells as previously described by Sadler et al., 1989. Isolated nuclei were attached to a polylysine-coated slide, fixed for 5 min in 1% formaldehyde, washed with 50 mM $NH_4Cl$ in PBS, 0.2% BSA in PBS, 0.1% Triton X-100 in PBS, and incubated with primary antibodies to the 70-kD NLS-binding protein, nucleolar proteins (from J. Broach, Princeton University), KAR2 (from M. Rose, Princeton University), and histone H2B (from M. Grunstein, University of California, Los Angeles, CA) diluted in buffer A with 1 mg/ml BSA, washed three times with buffer A/BSA, incubated with FITC-conjugated goat anti-mouse (Miles Scientific Div., Naperville, IL) or anti-rabbit antibody (Sigma Chemical Co.) diluted 1:1,000 in buffer A/BSA, washed three times with buffer A/BSA and once with buffer A/BSA with DAPI (1 μg/ml). Cells and nuclei were viewed at 1,000× with an Axioskope equipped for fluorescence (Carl Zeiss, Inc., Thornwood, NY). In control experiments, cells and nuclei showed no reactivity with the FITC-conjugated antibodies or with preimmune IgG.

## Gel Electrophoresis and Immunoblotting

Proteins were separated by SDS-PAGE in gels containing 7.5–15% gradients of acrylamide. For separation of nucleolar proteins and histones, gradients of 10–20% acrylamide were used.

After electrophoresis, proteins were blotted to nitrocellulose and filters incubated with 5% nonfat dry milk in PBS (1 h/RT). Filters were subsequently incubated 12 h at 4°C with antibodies diluted in 5% nonfat dry milk/PBS and washed with 0.1% Tween-20 in PBS. Bound antibodies were detected with secondary antibodies coupled to alkaline phosphatase. Antibodies directed against the 70-kD NLS-binding protein were visualized with secondary antibodies coupled to HRP and an enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL).

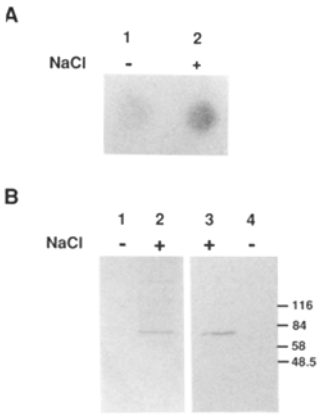


*Figure 1.* Analysis of the 70-kD NLS-binding protein: correlation between dot blot, gel binding assay, and Western blotting. Equal amounts of nuclei were incubated with buffer A in the absence or presence of 0.3 M NaCl as indicated in the figure. NLS-binding activity liberated from the nuclei by this treatment (see Materials and Methods) was determined by the dot-blot assay (*A*) and the gel binding assay (*B*, lanes *1* and *2*). In parallel, the presence of the 70-kD NLS-binding protein was analyzed by Western blotting (*B*, lanes *3* and *4*). For the dot blot and gel binding assays, nitrocellulose filters were incubated with 0.1 $\mu$Ci/ml $^{125}$I-SV40-HSA as detailed in Materials and Methods. Detection of anti-70-kD antibodies was carried out using secondary antibodies coupled to HRP followed by incubation with luminol in the presence of hydrogen peroxide.

## Results

We have characterized the role of a yeast protein of ~70 kD that specifically recognizes localization sequences in proteins for nuclear import. These experiments are dependent on assays for NLS binding, purification of the 70-kD protein, as well as generation of antibodies.

### *Assays for NLS-binding Proteins*

Proteins destined for the nuclear interior may interact with specific receptors via their nuclear localization sequences. In fact, yeast nuclei contain at least two such proteins of 70 and 59 kD that specifically recognize nuclear localization sequences. These proteins were originally identified by the binding of native nuclear proteins, as well as NLS-HSA conjugates to proteins immobilized on nitrocellulose following gel electrophoresis (Silver et al., 1989).

To circumvent the denaturation and possible loss of function during SDS-gel electrophoresis, we developed a rapid "dot-blot" assay. Yeast extracts were adsorbed directly onto nitrocellulose filters and then probed with the ligand $^{125}$I-SV40-HSA. The association of $^{125}$I-SV40-HSA correlated with the presence of the NLS-binding proteins as determined by gel electrophoresis followed by transfer to nitrocellulose. Extraction of yeast nuclei with buffer containing 0.3 M salt liberated the 70-kD NLS-binding protein (Fig. 1 *B*, lane 2). The same sample gave a positive signal by the dot-blot assay (Fig. 1 *A*, lane 2). Extracts made in the absence of salt contained little NLS-binding protein as determined by either method (Fig. 1, *A* and *B*, lanes *1*). Extracts heated to 95°C for 10 min showed no binding activity by the dot-blot assay (data not shown), consistent with NLS-binding activity being inactivated by heat denaturation.

### *Purification of NLS-binding Proteins*

We have purified the 70-kD NLS-binding protein and now show that the native protein can recognize NLS in the absence of nuclei, ATP, and other proteins. NLS-binding proteins were purified from salt extracts of yeast nuclei. As demonstrated above (Fig. 1 *B*, lane 2), NLS-binding proteins are

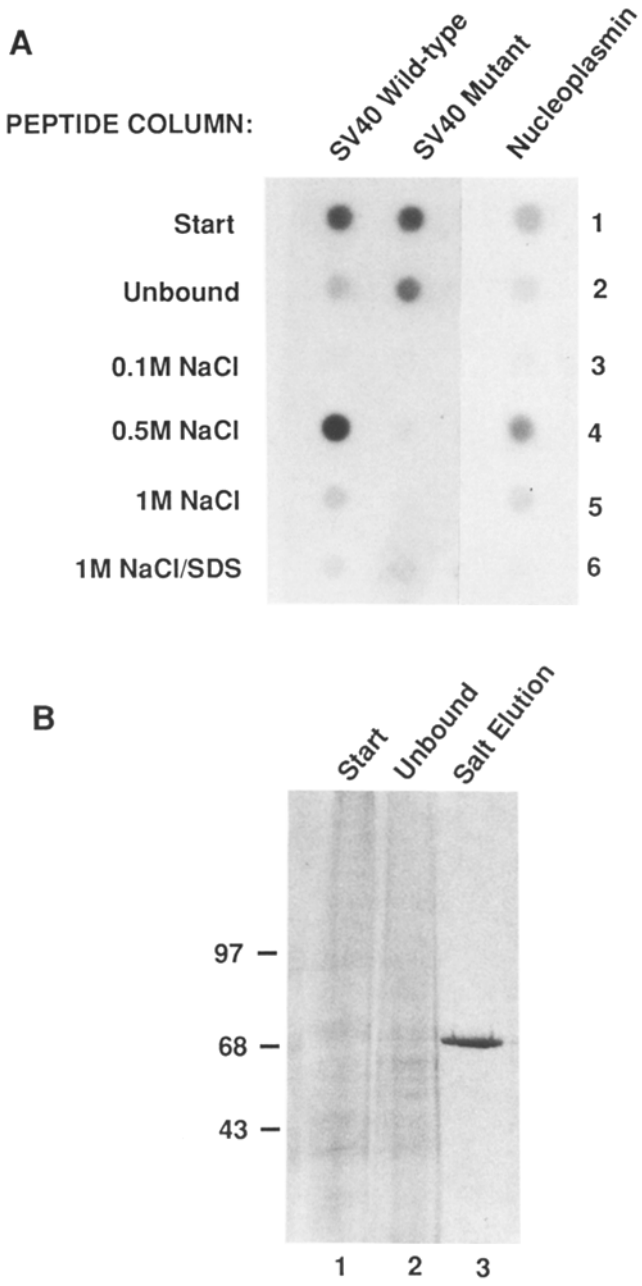


*Figure 2.* Binding of NLS-binding proteins to immobilized nuclear localization sequences. (*A*) Salt elution of NLS-binding activities. Affinity resins bearing the SV40 wild-type or mutant NLS or nucleoplasmin-HSA were prepared as described in Materials and Methods. Equal amounts of wild-type and mutant peptides were coupled to the matrices. Yeast extracts were chromatographed on DEAE-Sepharose and subsequently incubated with the different affinity resins. Affinity resins were washed with buffer A and eluted with increasing concentrations of NaCl or buffer containing 1 M NaCl with 0.2% SDS as indicated. Comparable amounts of the starting material, unbound and eluted proteins were analyzed for NLS-binding activity by the dot-blot assay. (*B*) Purification of the 70-kD NLS-binding protein by incubation with immobilized SV40 NLS. Yeast proteins were incubated with SV40 NLS-resin as described (*A*). Equal amounts of the starting material (lane *1*) and unbound proteins (lane *2*) were subjected to electrophoretic separation followed by silver staining of the gel to visualize proteins. Salt-eluted proteins (~400-fold concentrated as compared to the starting material) were analyzed in parallel (lane *3*). Numbers on the left indicate molecular weights $\times 10^{-3}$ of marker proteins.

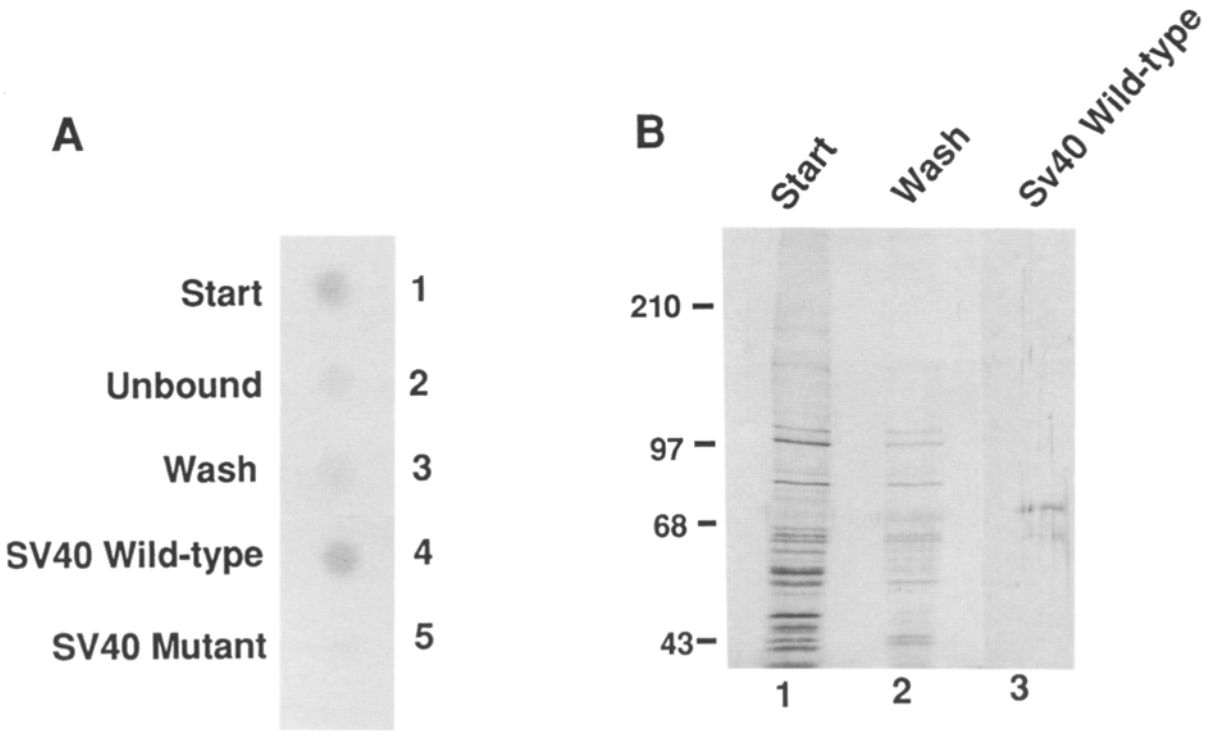


*Figure 3.* Affinity purification of NLS-binding proteins. (*A*) Binding of NLS-binding proteins to immobilized NLS. Proteins liberated from nuclei by extraction with 0.3 M NaCl were incubated with immobilized SV40 wild-type NLS in buffer A/50 mM NaCl. After washing with the same buffer, the resin was incubated with buffer A/50 mM NaCl and 1 mM free SV40 wild-type or SV40 mutant peptide. NLS-binding activities present in the starting material (row *1*), unbound (row *2*), recovered after washing (row *3*), released with SV40 wild-type (row *4*) and SV40 mutant peptide (row *5*) were determined by the dot-blot assay. Comparable amounts of each fraction were analyzed in parallel. (*B*) Purification of the 70-kD NLS-binding protein. Material obtained after extraction of nuclei with buffer A/0.3 M NaCl were incubated with immobilized SV40 NLS as described in *A*. Aliquots of the starting material (lane *1*), proteins recovered after washing the resin (lane *2*), and liberated by the SV40 wild-type peptide (lane *3*) were separated by SDS-PAGE and visualized by silver staining of the gel. Molecular weights of marker proteins $\times 10^{-3}$ are indicated on the left.

liberated from yeast nuclei by 0.3 M salt. These salt extracts were subjected to anion exchange chromatography (data not shown). NLS-binding proteins bound to DEAE sepharose and were eluted from the resin at ~300 mM salt. However, the active fractions still contained a large number of different proteins (Fig. 2 *B*, lane *1*).

To further purify the NLS-binding proteins, we have taken advantage of their affinity for nuclear localization sequences. Affinity resins were developed that contained either the wild-type or mutant NLS from SV40 T-antigen or the NLS from *Xenopus* nucleoplasmin covalently linked to sepharose (see Materials and Methods). (The nucleoplasmin NLS has also been shown to target proteins to the nucleus in *S. cerevisiae* [Silver et al., 1989; Sadler and Silver, unpublished results].) Fractions obtained after anion exchange chromatography that contained NLS-binding proteins were incubated with the different affinity resins. Most of the NLS-binding proteins remained bound to the wild-type SV40 or the nucleoplasmin NLS-resins as determined by the dot-blot assay (Fig. 2 *A*, compare rows *1* and *2*). On the other hand, very little protein remained associated with the resin containing the mutant SV40 NLS (Fig. 2 *A*, rows *1* and *2*), demonstrating the specific recognition of functional nuclear localization sequences under the conditions used.

Efficient release of the NLS-binding proteins from the wild-type SV40 or nucleoplasmin NLS-containing resins was obtained by elution with 0.5 M NaCl (Fig. 2 *A*, row *4*). A single polypeptide (identified by silver stain) of 70 kd molecular mass was recovered after elution of the wild-type SV40 NLS resin with 0.5 M NaCl (Fig. 2 *B*, lane *3*). The 59-kD protein was also occasionally recovered from the column in the same fraction as the 70-kD protein. Further washing of the column with SDS and 1 M NaCl did not release any additional proteins (Fig. 2 *A*, row *6*).

The specificity of the SV40 NLS affinity resin was further demonstrated by elution with free NLS peptides. As described above, fractions obtained from anion exchange chromatography contained proteins that bound to the SV40 NLS-containing resin (Fig. 3 *A*, rows *1* and *2*). Subsequent incubation of the resin with 1 mM free wild-type SV40 NLS peptide liberated NLS-binding activity as determined by the dot-blot assay (Fig. 3 *A*, row *4*). As observed with salt elution of the SV40 NLS affinity resin, the 70-kD protein was the predominant polypeptide eluted from the resin by the wild-type peptide (Fig. 3 *B*, lane *3*). In contrast, incubation of the wild-type NLS-resin with the mutant peptide did not release NLS-binding activity (Fig. 3 *A*, row *5*) and no 70-kD protein was detected in the eluant (data not shown).

Since the 70-kD NLS-binding protein is similar in size to the previously identified 70-kD heat shock proteins, we tested the possibility that these proteins were identical. However, antibodies directed against KAR2, the yeast analogue

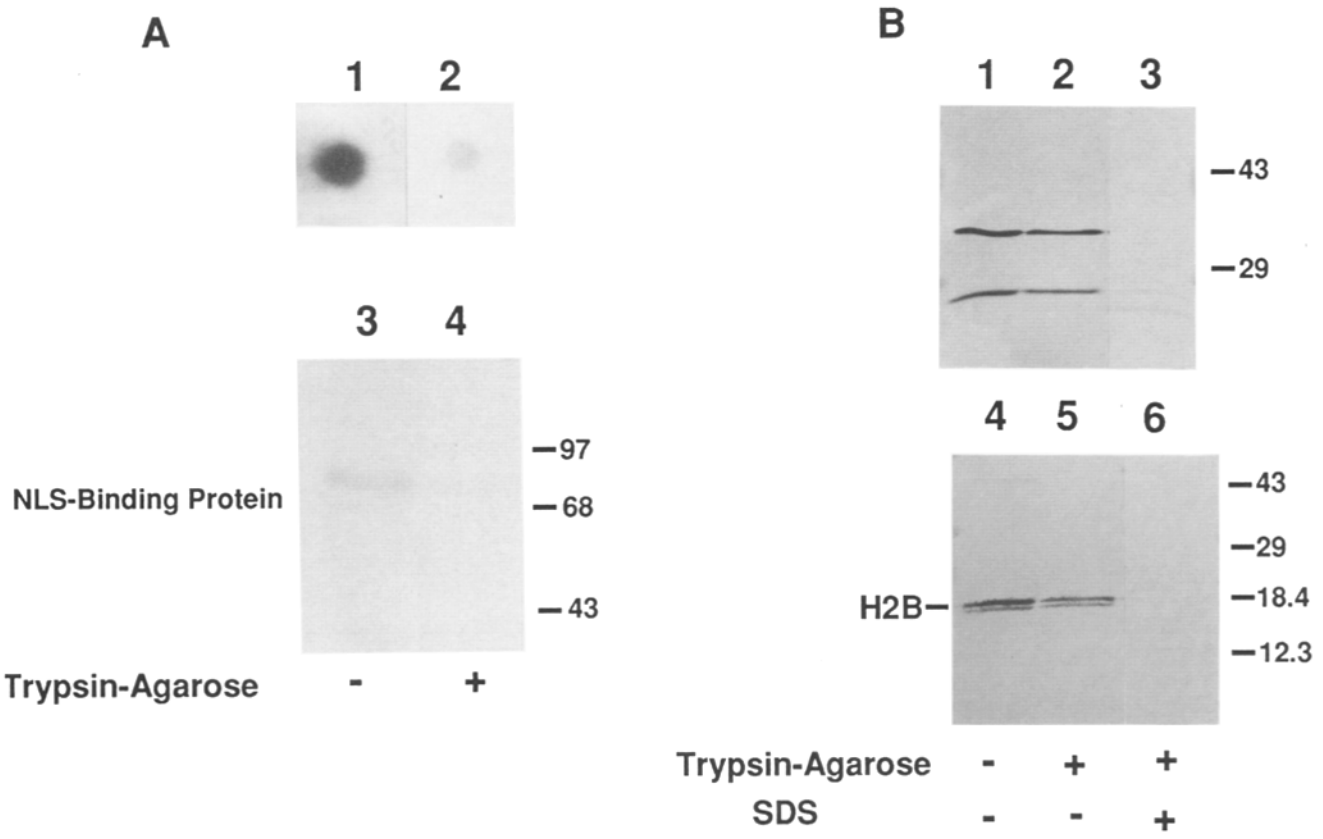


*Figure 4.* Incubation of nuclei with trypsin-agarose. (*A*) Loss of NLS-binding activities after treatment with trypsin-agarose. Nuclei were incubated in the absence (dot 1, lane *3*) or the presence (dot 2, lane *4*) of trypsin-agarose as described in Materials and Methods. After termination of the proteolysis, NLS binding was determined by the dot-blot assay (*1* and *2*) or by the gel binding assay (*3* and *4*). NLS-binding activities detected by the dot-blot assay corresponded to the 70- and 59-kD NLS-binding proteins (lane *3*). Molecular weights $\times 10^{-3}$ of marker proteins are indicated on the right. (*B*) Accessibility of nucleolar proteins and histone H2B to trypsin-agarose. Nuclei were incubated in the absence (lanes *1* and *4*) or presence (lanes *2*, *3*, *5*, and *6*) of trypsin-agarose as described in *A*. Samples shown in lanes 3 and 6 were treated with trypsin-agarose in the presence of 0.2% SDS. After treatment with trypsin, proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters. The filters were probed with antibodies directed against nucleolar proteins (lanes *1–3*) and histone H2B (lane *4–6*). Bound antibodies were visualized with secondary antibodies coupled to alkaline phosphatase. Molecular weights $\times 10^{-3}$ of marker proteins are shown on the right side.

of BiP (Rose et al., 1989) did not react with the 70-kD NLS-binding proteins. Similarly, antibodies to HSP70 proteins (Chappell et al., 1986) did not recognize the 70-kD NLS-binding protein. Moreover, analysis of the various fractions from the SV40 NLS affinity resin demonstrated that KAR2 (BiP) did not bind to the wild-type SV40 NLS-resin under the conditions used in our experiments (data not shown).

Taken together, these results show that NLS-binding proteins are associated with yeast nuclei, but can be extracted by intermediate salt concentrations (0.3 M NaCl). Binding to nuclear localization sequences is specific as evidenced by the lower affinity of the proteins for the mutant SV40 NLS as compared to that for the wild-type SV40 NLS-resins. Moreover, the ability of purified proteins to bind NLS indicates that the recognition of nuclear localization sequences by the native proteins does not require their association with the nucleus or additional factors.

### *Subcellular Location of NLS-binding Proteins*

We have carried out two different types of experiments to learn more about the intracellular location of the 70-kD NLS-binding protein. The results of the first set of experiments show that NLS-binding activity is accessible to trypsin conjugated to agarose beads under conditions where proteins of the nuclear interior are not affected. Yeast nuclei were incubated with trypsin conjugated to agarose beads of 45–165 $\mu$m in diameter, which are too large to penetrate the nuclear pore (Peters, 1986). Treatment of nuclei with trypsin-agarose resulted in elimination of NLS-binding proteins as determined by both the dot-blot assay (Fig. 4 *A*, compare lanes *1* and *2*) and the gel binding assay (Fig. 4 *A*, lanes *3* and *4*). Under the same conditions, two nucleolar proteins (Fig. 4 *B*, lanes *1* and *2*) and histone H2B (Fig. 4 *B*, lanes *4* and *5*) were not affected by trypsin-agarose. However, permeabilization of nuclei with 0.2% SDS (Fig. 4 *B*, lanes *3* and *6*), 0.5% Triton X-100, or freeze-thaw lysis (data not shown), rendered these proteins accessible to proteolytic degradation. Taken together, these results suggest that protease-sensitive sites of the 70-kD NLS-binding protein are exposed on the nuclear surface.

For the second set of experiments, antibodies were generated against the purified 70-kD NLS-binding protein (see Materials and Methods) and used to analyze yeast cell ex-

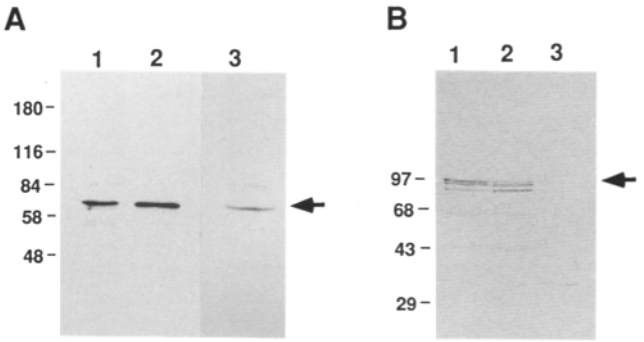


*Figure 5.* Identification of the 70-kD NLS-binding protein and proteins recognized by antibodies directed against the Nspl peptide. Isolated yeast nuclei (*A* and *B*, lanes *1*), proteins liberated from nuclei with 0.3 M NaCl (*A* and *B*, lanes *2*), and yeast cytosol (*A* and *B*, lanes *3*) were separated by SDS-PAGE and blotted on to nitrocellulose. Each lane contained 50 $\mu$g of protein. Filters were blocked and subsequently incubated with antibodies directed against the 70-kD NLS-binding protein (*A*) or affinity-purified antibody against the peptide CSKPAFSFGAK. Antibodies against the 70-kD NLS-binding protein were detected with a secondary antibody coupled to HRP followed by incubation with luminol in the presence of hydrogen peroxide. Antibodies against the Nspl-peptide were visualized with secondary antibodies conjugated to alkaline phosphatase. Molecular weights $\times 10^{-3}$ are indicated in the margins of *A* and *B*. The position of the 70-kD NLS-binding protein and nucleoporins are indicated by the arrow.

tracts. The antibodies recognized the 70-kD protein associated with yeast nuclei (Fig. 5 *A*, lane *1*). As expected, the 70-kD proteins recognized by the antibodies cofractionate with nuclei, and can be released with 0.3 M NaCl (Fig. 1 *B*, lane *4* and Fig. 5 *A*, lane *2*), as we have shown for the NLS-binding proteins. In addition, yeast cytosolic fractions also contain 70-kD NLS-binding protein (Fig. 5 *A*, lane *3*). Analysis of cell fractions by quantitative immunoblotting indicated that ~70% of the total 70-kD NLS-binding protein was associated with the nuclei-containing fraction. 20% of the NLS-binding protein was in the cytosol-containing fraction. The remaining protein was found in a mitochondrial-enriched fraction that was contaminated with nuclei.

Immunofluorescence with the anti-70-kD antibody demonstrated that the antigen is nuclear associated. Fixed and permeabilized whole yeast cells were probed with the anti-70-kD NLS-binding protein followed by a secondary FITC-conjugated antibody. Staining with the anti-70-kD antibody was concentrated mainly at the nucleus with some additional cytoplasmic staining observed (Fig. 6, *A–C*). These findings are consistent with the results obtained with immunoblotting of subcellular fractions (see above).

To further assess the location of the NLS-binding proteins, immunofluorescence was carried out on isolated yeast nuclei. We compared the staining pattern obtained for the 70-kD protein to those of other nuclear-associated proteins.

Antibodies recognizing proteins of the yeast nucleoporins were generated as follows. Recently, two yeast nucleoporins, Nsp1 and Nup1, were shown to contain a common stretch of amino acid residues (Davis and Fink, 1990; Nehrbass et al., 1990). We raised antibodies against the peptide CSKPAFSFGAK, corresponding to these conserved residues. Western blotting of yeast nuclear proteins revealed that the affinity-purified antibodies bound to several proteins with an apparent molecular mass of 100 kD (Fig. 5 *B*, lane *1*). A similar pattern is observed with anti-Nsp1 antibodies (Hurt, 1988), suggesting that a family of related proteins was recognized by the peptide-specific antibodies. Some of these proteins were extractable from nuclei by salt (Fig. 5 *B*, lane *2*) and were not present in a cytosolic fraction (Fig. 5 *B*, lane *3*). Immunofluorescence of whole yeast cells (data not shown) and isolated nuclei (Fig. 6 *B*, *A–C*) using the peptide antibody revealed "punctate" staining of the nuclear envelope as has been observed with anti-Nsp1 antibodies (Nehrbass et al., 1990), confirming the reactivity of this antibody with nuclear pores. Thus, these antibodies directed against the synthetic peptide were suitable markers for nuclear pores.

The anti-70-kD antibodies reacted with isolated nuclei. Staining of the entire nucleus was observed (Fig. 6 *B*, *J–L*). The staining pattern obtained for the anti-70-kD antibodies differed from those obtained with antibodies directed against yeast nucleolar proteins (Fig. 6 *B*, *M–O*), histone H2B (Fig. 6 *B*, *G–I*) or KAR2 (Fig. 6 *B*, *D–F*). In sum, these data support the nuclear association of a major portion of the 70-kD NLS-binding proteins.

### *Antibody Inhibition and Reconstitution of NLS Binding Activity*

The NLS-binding proteins are defined by their ability to specifically interact with nuclear localization sequences. We have previously shown that $^{125}$I-SV40-HSA specifically associates with yeast nuclei in vitro (Silver et al., 1989). To analyze whether the 70-kD NLS-binding protein is an important component for this nuclear association, we tested the effect of anti-70-kD antibodies on the nuclear binding of $^{125}$I-SV40-HSA. Nuclei were preincubated with increasing concentrations of antibodies directed against the 70-kD NLS-binding protein followed by addition of the $^{125}$I-SV40-HSA substrate. In parallel, the effect of antibodies against the Nsp1 repeat peptide or *E. coli* $\beta$-galactosidase was examined. As shown in Fig. 7, antibodies against the 70-kD NLS-binding protein reduced binding of $^{125}$I-SV40-HSA to nuclei to <20% of their original activity (Fig. 7, *open circles*). Inhibition of binding was dependent on the concentration of antibody. Anti-70-kD antibodies did not recognize SV40-HSA (data not shown), which rules out the trivial explanation that inhibition of nuclear binding was due to inactivation of the substrate.

The anti-Nsp1 peptide antibody inhibited SV40-HSA binding at higher antibody concentrations than that for the anti-70-kD antibody (Fig. 7, *diamonds*). The inhibition was also dependent on antibody concentration; at low antibody concentration there was no effect on binding. The anti-Nsp1 peptide antibody does not react with the 70-kD NLS-binding protein or with SV40-HSA. Under identical conditions, antibodies against $\beta$-galactosidase had no effect on SV40-HSA binding to nuclei.

We show that removal of NLS-binding proteins from the nucleus interferes with SV40-HSA binding. Moreover, readdition of NLS-binding protein-enriched extracts to partially inactivated nuclei restored their ability to bind NLS-containing proteins. Taken together with the antibody inhibition results, these data suggest that the 70-kD NLS-binding protein

could be important for the initial binding of imported proteins to nuclei. Further experiments are required to define in more detail its role in nuclear protein import.

Gentle treatment of nuclei with either trypsin-agarose or NaCl reduced binding of SV40-HSA to ~50% of the untreated controls (Fig. 8 *A*), consistent with the partial extraction of NLS-binding proteins from nuclei. High concentrations of salt or prolonged incubation with proteases allowed total elimination of the 70-kD NLS-binding proteins but resulted in lysis of the nuclei. Salt or trypsin-treated nuclei still specifically bound TRITC-SV40-HSA at a reduced extent; mutant TRITC-SV40-HSA and TRITC-HSA still remained unbound (data not shown). Association of SV40-HSA can be restored to salt- or trypsin-treated nuclei by addition of extracts enriched for NLS-binding proteins. Moreover, preincubation of nuclei with salt extracts resulted in a similar restoration of SV40-HSA nuclear association. Proteins further purified by anion exchange chromatography and 500-fold enriched for NLS-binding activity could also partially restore binding to salt-treated nuclei (Fig. 8 *A*). Incubation of salt-treated nuclei with extracts containing NLS-binding proteins resulted in reassociation of the 70-kD NLS-binding protein with nuclei, which could be detected by Western blot (Fig. 8 *B*, lanes *3* and *4*). In summary, the reassociation of salt-extracted components, including the 70-kD NLS-binding proteins, permitted reconstitution of the binding and was almost complete for salt-washed nuclei.

To further demonstrate the importance of the 70-kD NLS-binding proteins for the reconstitution of nuclear binding of SV40-HSA, we depleted extracts for the NLS-binding protein. Nuclear extracts were depleted for the 70-kD NLS-binding protein by passage over a column carrying covalently attached antibodies to the 70-kD protein. These immunodepleted extracts were subsequently used for reconstitution of the binding reaction. As shown in Fig. 8 *A*, depletion of the 70-kD NLS-binding protein resulted in loss of the ability of the extract to reconstitute nuclei with binding activity. However, gel electrophoresis followed by silver staining of the material bound to the column revealed that proteins other than the 70-kD NLS-binding protein were also being retained (data not shown), perhaps due to their association with the NLS-binding protein. Some of these proteins might also be important for restoration of binding activity to nuclei or might represent nuclear proteins transiently associated with the 70-kD NLS-binding protein.

## Discussion

Nuclear protein import is a multi-step process (Silver, 1991). Binding of NLS-containing proteins at the nuclear pore complex occurs before transit of proteins into the nucleus (Newmeyer and Forbes, 1988; Richardson et al., 1988). The yeast, *Saccharomyces cerevisiae* contains proteins that specifically interact with nuclear localization sequences (Silver et al., 1989; Lee and Melese, 1989). We have used the specificity of the interaction between these NLS-binding proteins and nuclear localization sequences to purify the 70-kD NLS-binding protein. The 70-kD NLS-binding proteins are important for nuclear protein uptake because (*a*) antibodies inhibit binding of SV40-HSA to nuclei; and (*b*) an extract enriched for the 70-kD NLS-binding protein can be added back to partially stripped nuclei to restore NLS-HSA binding.

### *Purification of NLS-binding Proteins*

The NLS-binding proteins can be purified from yeast nuclei by affinity chromatography based on their interactions with nuclear localization sequences. In particular, the 70-kD protein bound only to affinity resins containing wild-type nuclear localization sequences and not to resins containing mutant forms of the NLS. In addition, the 70-kD protein was specifically eluted from the wild-type NLS-containing resin by wild-type SV40 NLS peptide, but not by mutant peptide. These results indicate that the native NLS-binding proteins are active even after release from nuclei and do not require the presence of nuclear envelope components for correct recognition of nuclear localization sequences. Since the proteins retain their NLS-binding activity after liberation from the nuclear surface, it is tempting to speculate that these polypeptides might exist transiently as free and active binding proteins in the cytoplasm to deliver proteins to the nucleus. This has been suggested by others who found similar proteins in the cytoplasm of mammalian cells (Adam et al., 1989; Yamasaki et al., 1989).

### *Nuclear Association of NLS-binding Proteins*

Others have shown that an early step in nuclear protein uptake is ATP-independent binding at the nuclear surface or the nuclear pore complex (Newmeyer and Forbes, 1988; Richardson et al., 1988; Akey and Goldfarb, 1989). Proteins mediating this binding would be expected to be at least transiently associated with the nuclear surface. We now show that proteins that can mediate NLS-dependent binding reside with the nucleus in a manner consistent with at least some of the protein being at the nuclear surface.

Two yeast proteins of 70 and 59 kD have been identified that interact with a number of different, but related, nuclear localization sequences (Silver et al., 1989). Both proteins are accessible to proteases that cannot penetrate the nuclear envelope by virtue of their size and failure to digest intranuclear proteins. Moreover, protease treatment of nuclei significantly reduced the binding of added SV40-HSA, thus, supporting the notion that proteins on the cytoplasmic face of the nuclear envelope are important for nuclear protein binding. Kalinich and Douglas (1989), also observed that SV40 T-antigen import was reduced if yeast nuclei were pretreated with proteases conjugated to beads. The NLS-binding proteins can also be removed from nuclei by salt treatment indicating that they are only peripherally associated with the nucleus possibly via ionic interactions. These data support at least a partial nuclear-surface association for the NLS-binding proteins.

We used antibodies to further analyze the intracellular distribution of the 70-kD NLS-binding protein. Western blot analysis of cell fractions confirmed that the NLS-binding protein is mostly nuclear associated with minor amounts in the cytoplasm.

Independent confirmation of these results was obtained by immunofluorescence. In whole cells, the 70-kD NLS-binding protein is mostly nuclear associated with some weaker staining in the cytoplasm. Immunofluorescence of isolated nuclei also indicated that the nature of the nuclear association of the 70-kD NLS-binding protein differed from proteins restricted to the nuclear interior (histone H2B and proteins of the nucleolus). No specific staining of the nucleolus was ob-

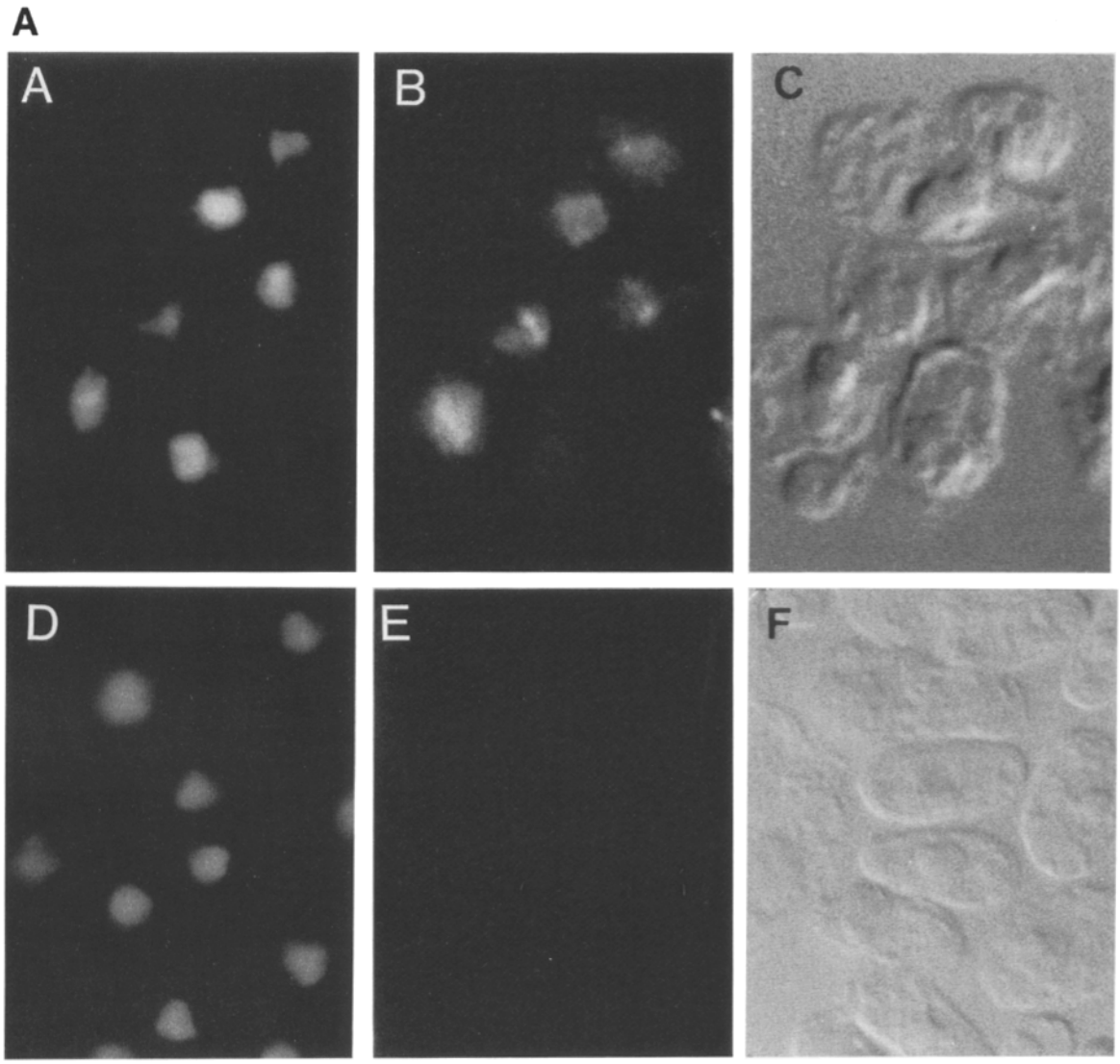


*Figure 6.* Localization of NLS-binding proteins by immunofluorescence. (*A*) Yeast cells were prepared for immunofluorescence as described in Materials and Methods, treated with mouse anti-70-kD antibody (*B*) or mouse preimmune IgG (*E*), followed by FITC-conjugated anti-mouse IgG, DAPI to visualize nuclei (*A* and *D*), and Nomarski to visualize cells (*C* and *F*). (*B*) Nuclei were prepared for immunofluorescence as described in Materials and Methods and treated with rabbit anti-Nspl peptide antibody (1:30) followed by FITC-conjugated anti-rabbit IgG (*C*), rabbit anti-KAR2 antibody (1:1,000) followed by FITC-anti-rabbit IgG (*D*), rabbit antihistone H2B antibody (1:500) followed by FITC-anti-rabbit IgG (*G*), mouse anti-70-kD antibody (1:100) followed by FITC-conjugated anti-mouse IgG (*L*), mouse antinucleolar antibody (1:1,000) followed by FITC-anti-mouse IgG (*M*), and preimmune mouse IgG (1:100) followed by FITC-conjugated anti-mouse IgG (*P*). *B*, *E*, *H*, *K*, *N*, and *Q* are nuclei stained with DAPI, and *A*, *F*, *I*, *L*, *O*, and *R* are nuclei viewed by Normarski.

served in contrast to what has been shown for the 140-kD rat liver NLS-binding protein, which is located exclusively at the nucleolus (Meier and Blobel, 1990).

Two yeast nuclear pore proteins, Nspl (Nehrbass et al., 1990) and Nupl (Davis and Fink, 1990), have been characterized. Cytologically, these proteins are associated with the nuclear pore (Aris and Blobel, 1989). Comparison to the localization of nucleoporins by immunofluorescence suggested that some NLS-binding protein (but not all) may be similarly distributed.

### *Possible Role of the 70-kD NLS-binding Protein in Nuclear Protein Import*

We used antibodies against the 70-kD NLS-binding protein to assess its role in assembly of proteins with nuclei in vitro. We reported previously that binding of $^{125}$I-SV40-HSA to nuclei was specific and saturable (Silver et al., 1989). We now show that binding of $^{125}$I-SV40-HSA is reduced when nuclei are preincubated with antibodies to the 70-kD NLS-binding protein, suggesting that the 70-kD protein is important for the binding step in nuclear import. This is further confirmed by reconstitution experiments. Salt treatment and limited proteolysis reduced the presence of the NLS-binding proteins and the association of NLS-HSA proteins with nuclei. Addition of a fraction enriched for NLS-binding proteins restored both the 70-kD NLS-binding protein and the NLS-binding activity to salt-treated nuclei. An extract immunodepleted for the 70-kD NLS-binding protein could not restore binding activity to salt-treated nuclei.

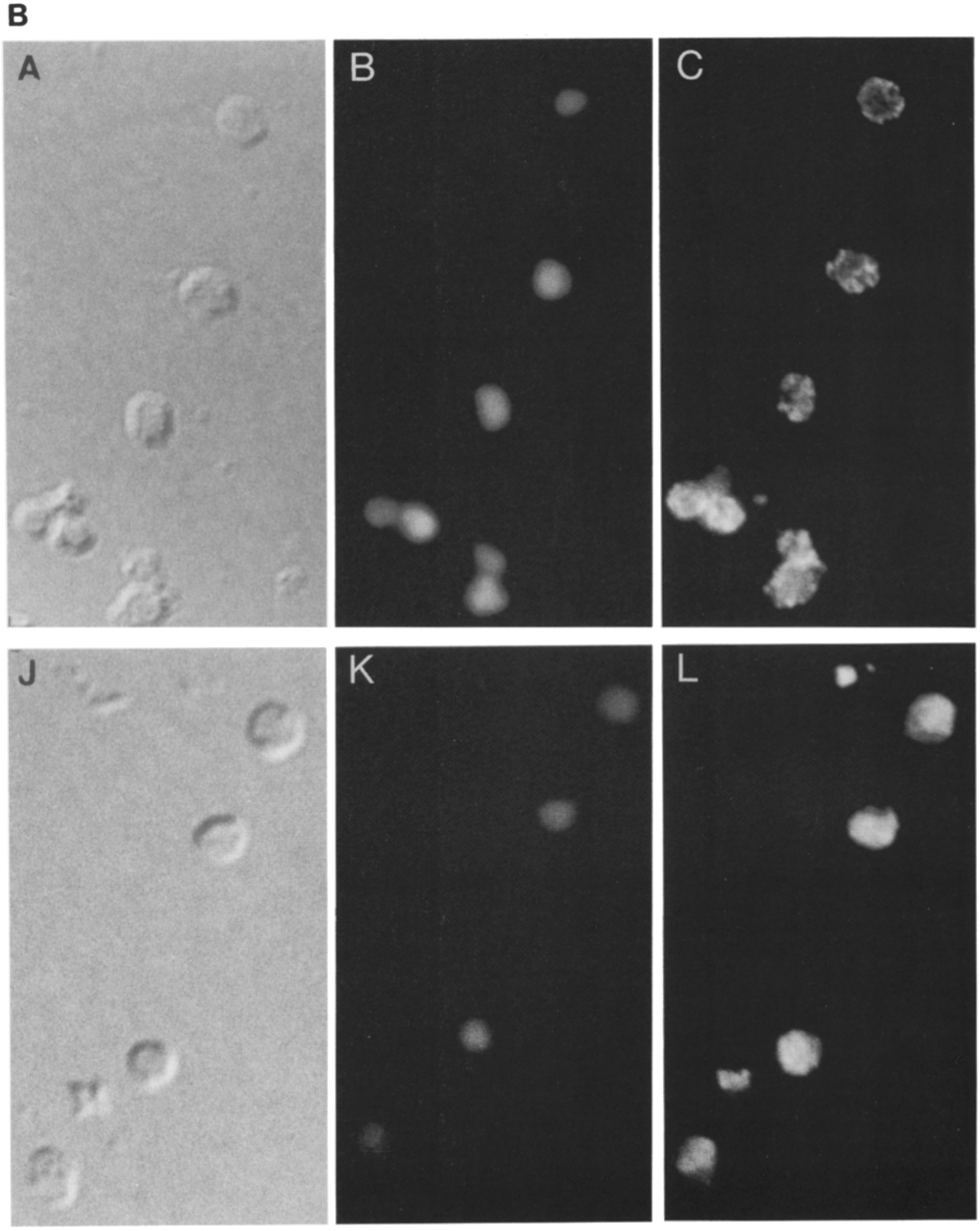


*Figure 6.*

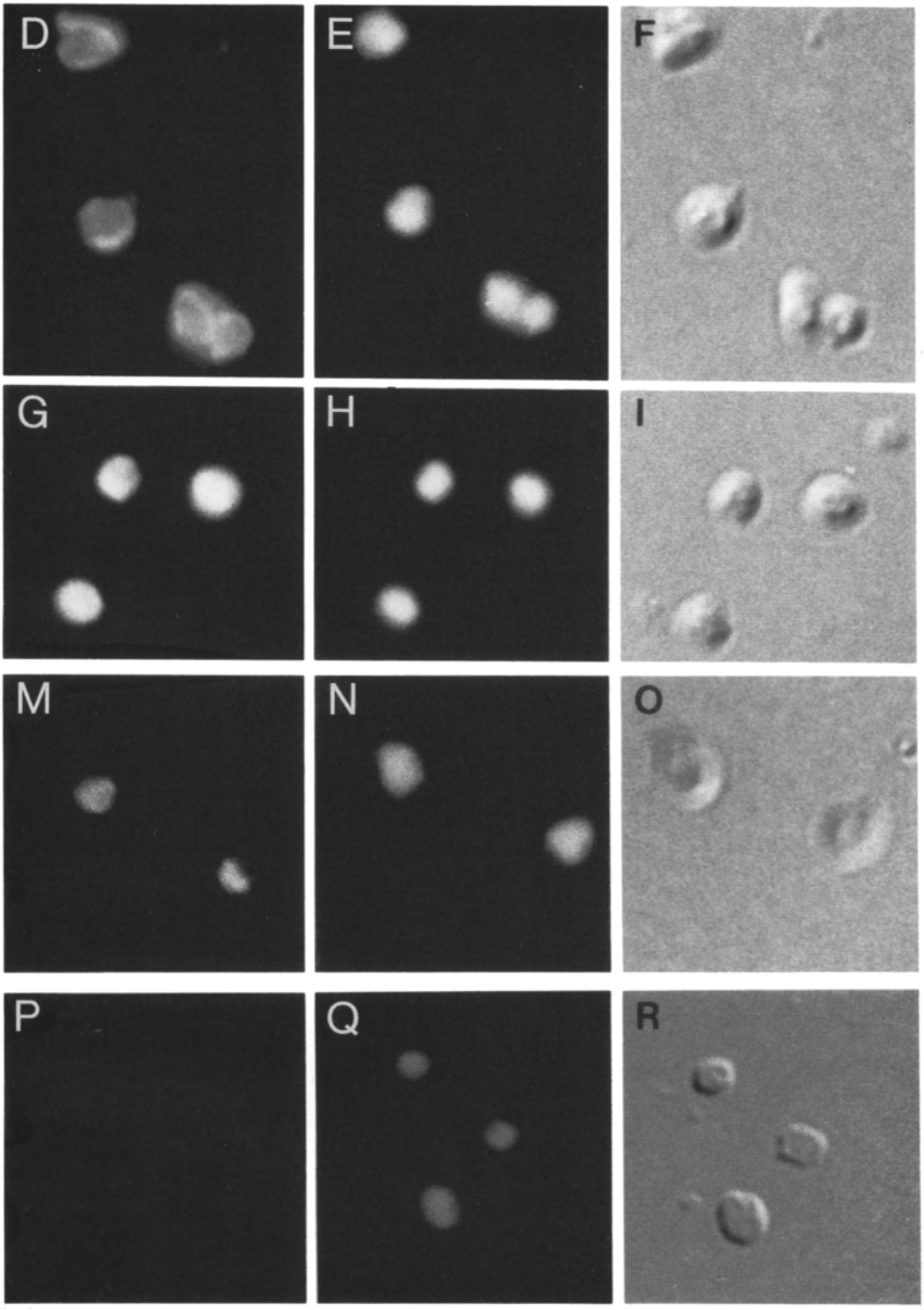


*Figure 6.*

Antibodies to the Nspl repeat peptide inhibited nuclear binding of SV40-HSA at higher concentrations than those for the 70-kD NLS-binding protein. There are several possible explanations for this effect. Nspl-like proteins may also mediate binding of nuclear-imported proteins. Alternatively, anti-Nspl peptide antibodies may disrupt the activity of the NLS-binding proteins by directly cross-reacting or by indirectly affecting the function of the NLS-binding proteins.

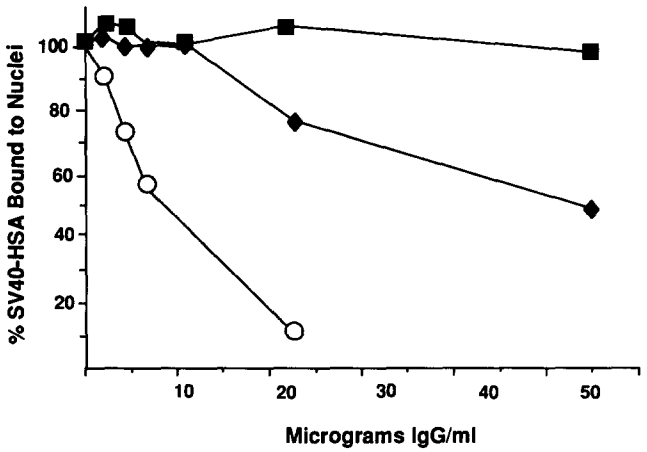


*Figure 7.* Effect of antibodies on NLS-dependent binding to nuclei. Yeast nuclei were preincubated with affinity-purified antibodies directed against the 70-kD NLS-binding protein (*open circles*), the Nspl repeat (*closed diamonds*), or β-galactosidase (*closed squares*) for 1 h at 4°C. Final antibody concentrations are depicted in the figure. Controls were incubated under identical conditions in the absence of antibodies. After preincubation, the binding of $^{125}$I-SV40-HSA was determined as detailed in Materials and Methods. The association of $^{125}$I-SV40-HSA to untreated controls was defined as 100% binding. Data shown are means of duplicate determinations. Two separate experiments gave similar results.

However, we do not see any cross-reactivity of these antibodies to NLS-binding proteins or to the SV40-HSA substrate.

We have presented a more detailed characterization of the 70-kD NLS-binding protein. The function of the 59-kD protein is not yet clear. Levels of the 59-kD protein vary and it is always much less abundant than the 70-kD protein.

Taken together, these results indicate that there is a correlation between the binding activity of nuclei and the presence of 70-kD NLS-binding proteins. They support the following model for nuclear protein import. Binding of nuclear proteins first occurs to their "receptors" present in the cytoplasm or on the nuclear surface, such as the 70-kD NLS-binding protein. Proteins are then released and translocated into the nucleus or cotransported with their receptors in subsequent reactions. The regulation of nuclear transport by changing the composition of the NLS-binding proteins offers a possible mechanism to control nuclear-cytoplasmic traffic in vivo. Alteration of the number and/or composition of receptors could modulate the efficiency of nuclear transport. Identification of the genes encoding the NLS-binding proteins and creation of yeast mutants in these genes will allow us to confirm aspects of this model.

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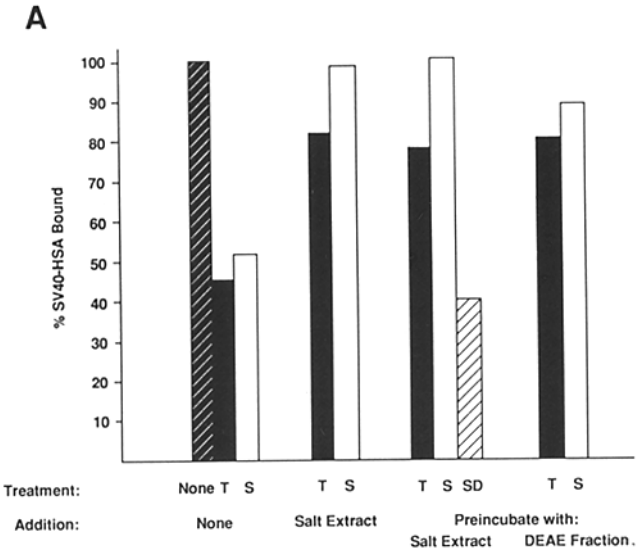


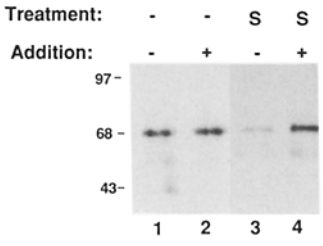


*Figure 8.* Reconstitution of nuclear protein association. (*A*) Nuclei were pretreated with trypsin-agarose (*T, black bars*) or 0.3 M NaCl (*S, white bars*) as described in Materials and Methods. Reconstitution of the binding reaction was obtained by addition of proteins released from nuclei with salt or partially purified by ion exchange chromatography (see Materials and Methods). Nuclei were incubated with these extracts for 30 min at 4°C before addition of the substrate $^{125}$I-SV40-HSA. Samples were kept for 15 min at 4°C before determination of the amount of radioactivity associated with nuclei. Alternatively, nuclei were preincubated for 30 min at 4°C with salt extracts, collected by centrifugation to remove unbound material, and incubated with $^{125}$I-SV40-HSA as described above. In parallel, nuclei were treated with salt and preincubated with extracts depleted of the 70-kD NLS-binding protein by passage over a column containing covalently bound antibodies directed against the 70-kD protein (*SD, striped bar*). Data were normalized to untreated controls, which are shown as 100% binding (no treatment, *striped bar*). Data shown are means of duplicate determinations that varied by 2%. Three separate experiments gave similar results. (*B*) Reassociation of 70-kD NLS-binding protein with salt-treated nuclei. Nuclei were preincubated in buffer A (lanes *1* and *2*) or buffer A containing 0.3 M NaCl (lanes *3* and *4*), collected by centrifugation and incubated in the presence (lanes *2* and *4*) or absence (lanes *1* and *3*) of salt extracts as described in *A*. Nuclei were subsequently collected by centrifugation, washed once with buffer A, and subjected to SDS-PAGE. Proteins blotted onto nitrocellulose were incubated with antibodies directed against the 70-kD NLS-binding proteins. Bound antibodies were detected with secondary antibodies coupled to HRP followed by incubation with luminol/hydrogen peroxide.

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