Reconstitution of Nuclear Protein Transport with Semi-Intact Yeast Cells

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Abstract. We have developed an in vitro nuclear protein import reaction from semi-intact yeast cells. The reaction uses cells that have been permeabilized by freeze-thaw after spheroplast formation. Electron microscopic analysis and antibody-binding experiments show that the nuclear envelope remains intact but the plasma membrane is perforated. In the presence of ATP and cytosol derived from yeast or mammalian cells, a protein containing the nuclear localization sequence (NLS) of SV40 large T-antigen is transported into the nucleus. Proteins with mutant NLSs are not imported. In the absence of cytosol, binding of NLS-containing proteins occurs at the nuclear envelope. *N*-ethylmaleimide treatment of the cytosol as well as antibodies to the nuclear pore protein Nspl inhibit import but not binding to the nuclear envelope. Yeast mutants defective in nuclear protein transport were tested in the in vitro import reaction. Semi-intact cells from temperature-sensitive *nspl* mutants failed to import but some binding to the nuclear envelope was observed. On the other hand, no binding and thus no import into nuclei was observed in semi-intact *nsp49* cells which are mutated in another nuclear pore protein. *Npl3* mutants, which are defective for nuclear protein import in vivo, were also deficient in the binding step under the in vitro conditions. Thus, the transport defect in these mutants is at the level of the nucleus and the point at which nuclear transport is blocked can be defined.

Many nuclear-destined proteins contain short stretches of amino acids (termed NLS[1] for nuclear localization sequences) that target the protein to the nucleus (for review see Silver, 1991; Goldfarb and Michaud, 1991; Nigg et al., 1991; Garcia-Bustos et al., 1991*a*). The process of nuclear protein uptake occurs in at least two steps; NLS-dependent binding at the nuclear envelope followed by ATP-dependent translocation through the nuclear pore complex (Newmeyer and Forbes, 1988; Richardson et al., 1988; Akey and Goldfarb, 1989). Because of the apparent saturability (Goldfarb et al., 1986) and specificity of NLS function (e.g., Kalderon et al., 1984), receptors that recognize NLS-bearing proteins were postulated to exist. Several candidate proteins have been found in both the cytosol and associated with the nucleus (Adam et al., 1989; Lee and Melese, 1989; Li and Thomas, 1989; Silver et al., 1989; Meier and Blobel, 1990; Imamoto-Sonobe et al., 1990; Stochaj et al., 1991; Stochaj and Silver, 1992). Recognition of nuclear proteins may also occur in the cytoplasm by binding to NLS-specific receptors (Breeuwer and Goldfarb, 1990; Adam et al., 1990).

In vitro reactions have demonstrated several requirements for import. These assays employ either reconstituted nuclei from *Xenopus* oocytes (for review see Newmeyer and Wilson, 1991) or permeabilized mammalian (Adam et al., 1990; Moore and Blobel, 1992) or *Drosophila* tissue culture cells (Stochaj and Silver, 1992). All reactions require nuclear pore complex proteins and the presence of cytosolic proteins. Import can be inhibited by antibodies against nucleoporins (Featherstone et al., 1988; Dabauvalle et al., 1988; Greber and Gerace, 1992). Moreover, the lectin WGA, which binds to glycoproteins of the pore complex, also inhibits import but not binding of NLS-containing proteins at the nuclear pore complex (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988; Wolff et al., 1988). Finlay and Forbes (1990) also found that morphologically intact pores could assemble from *Xenopus* egg extracts depleted of pore glycoproteins but no specific transport through these pores occurred. For the cytosol requirement, factors sensitive to *N*-ethylmaleimide (NEM) treatment (Newmeyer and Forbes, 1990; Adam et al., 1990; Moore and Blobel, 1992) and with NLS-binding activity (Adam and Gerace, 1991; Stochaj and Silver, 1992) have been described. Finally, cytosolic hsp70s have also been implicated as important for transit of proteins into the nucleus (Shi and Thomas, 1992; Imamoto et al., 1992).

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1. *Abbreviations used in this paper*: DAPI, 4′,6′-diamidino-2-phenylindole; HSA, human serum albumin; MBS, maleimidobenzoyl-N-hydroxysuccinimide ester; NEM, N-ethylmaleimide; NLS, nuclear localization sequences.

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Studies with the yeast *Saccharomyces cerevisiae* have also been useful in defining components of the pore complex, as well as factors possibly involved in the transport process (for review see Bossie and Silver, 1992). For the pore complex, Nspl and Nupl were the first yeast nucleoporins to be described (Hurt, 1988; Davis and Fink, 1990; Nehrbass et al., 1990). They share a common repetitive motif of a nonapeptide in their middle domains and are similar to their mammalian counterpart, p62 (Carmo-Fonseca et al., 1991). Nspl is essential for cell growth and its depletion results in a defect in nuclear protein import (Mutvei et al., 1992). More recently, a new family of yeast nuclear pore complex proteins including Nsp49, has been defined by the presence of the internal repeat GLFG (Wente et al., 1992; Wimmer et al., 1992).

Using a genetic approach, this laboratory has identified *npl* mutants that are defective in nuclear protein localization (Sadler et al., 1989; Bossie et al., 1992; Bossie and Silver, 1992). *Npll* mutants may disrupt the integrity of the nuclear envelope whereas others such as *npl3* and *npl6* block import of NLS-bearing proteins into the nucleus (Bossie et al., 1992; Chiang, A., and P. A. Silver, unpublished results). A similar approach has yielded the yeast mutant *nipl* which is also blocked in nuclear protein import (Gu et al., 1992).

Because of the emerging genetic analysis with yeast (for review see Osborne and Silver, 1993), we have developed a reconstituted nuclear protein import reaction from *S. cerevisiae*. The goal of this approach is not only to define additional components biochemically, but also to analyze the nature of the defects in various known nuclear transport mutants. We have chosen to use a procedure previously shown to be successful for the study of the yeast secretory pathway (Baker et al., 1988; Ruohola et al., 1988) and vacuole division (Conradt et al., 1992)—the generation of semi-intact cells from yeast spheroplasts. We show that the requirements for nuclear transport with semi-intact yeast are similar to those observed for mammalian cells and *Xenopus* oocytes. Import of NLS-containing proteins requires cytosolic proteins and ATP, and is inhibited by NEM treatment of the cytosol. In the absence of cytosol and/or ATP, only binding at the nuclear envelope is observed. We show that import, but not binding, depends on the nucleoporin Nspl. On the other hand, mutations in *NPL3* and *NSP49* disrupt both binding and import.

Materials and Methods

Yeast Strains

For preparation of semi-intact cells, the following strains of *S. cerevisiae* were used: JU4-2 X JR26-19B (wild-type; [*MATa/α ade2-1/ade2-1 ade8/ADE8 canl-100*/canl-100 his4/HIS4 *his3/HIS3 leu2-3/leu2-3 lysl-1/lysl-1 ura3-52/ura3-52*]). Mutant strains included *nspl-10A* (*MATa ade2 ade8 leu2 lysl URA3::nspl^{ts}* His$^-$ [derived from JU4-2 X JR26-19B; Nehrbass et al., 1990]); nopl-3 (*MATa ade2 ade8 leu2 lysl URA3::nopl-3* [Tollervey et al., 1993]); *nsp49-3/13 (MATα ade2 ade3 his3 leu2 ura3 TRPl::nsp49 pUN90::HIS3-nsp49-3/13* [Doye, V., and E. C. Hurt, unpublished results]); PSY361 (*MATa ura3-1 leu2-3,112 trpl-1 ade2-1 lys2 npl3-1^{ts}* [Bossie et al., 1992]). Wild-type cytosol was prepared from ABYS1 (*MATa pral prbl prcl cpsl ade$^-$*).

Preparation of HSA-Conjugates

Peptides conjugated to human serum albumin (HSA) were prepared as follows. 20 mg of HSA (Calbiochem Corp., La Jolla, CA) were dissolved in 4 ml PBS and mixed with 1 ml of 12 mg/ml of maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Pierce, Rockford, IL) in dimethyl formamide (DMF). Unbound MBS was removed by passage over a 50-ml Sephadex G25 column equilibrated with 100 mM sodium phosphate pH 6.0. The HSA-MBS-containing fractions (6.6 ml) were pooled and aliquots of 2 ml were mixed with 1 ml of a 5-mg/ml solution of either wild-type (CTPPKKKRKV) or mutant (CTPPKTKRKV) NLS peptides. After overnight incubation at RT, 2-mercaptoethanol was added to a final concentration of 14 mM, and the reactions were dialyzed for 2 h against 100 mM NH_4HCO_3/250 mM NaCl, pH 8.0, followed by overnight dialysis against 50 mM sodium carbonate pH 9.1. To fluorescently tag HSA and the peptide-HSA conjugates, 5 mg of protein were dissolved in 3 ml 50 mM sodium carbonate, mixed with 12.5 μl lissamine rhodamine sulfonyl chloride (Molecular Probes, Eugene, OR; 20 mg/ml in DMF) and incubated for 1 h in the dark at 4°C with gentle agitation. Free dye was removed by passage over 20 ml Sephadex G25 columns equilibrated with buffer A (0.25 M sorbitol, 20 mM Pipes-KOH pH 6.8, 150 mM K-Acetate, 5 mM Mg-Acetate). The conjugates were further purified by passage over 1 ml Biobeads (Bio Rad Labs., Hercules, CA) columns and stored frozen. 20 wild-type NLS and 30 mutant NLS peptides were estimated to be coupled to HSA as judged by mobility shifts on SDS-PAGE.

Preparation of Semi-Intact Cells

Semi-intact cells were prepared as previously described with minor changes (Baker et al., 1988; Conradt et al., 1992). Cells were grown at 30 (wild-type) or 23°C (temperature-sensitive mutants) in 500 ml YPD to ≈1 × 10^7 cells/ml. For temperature-sensitive mutants, cells were shifted at OD_{600} of ~0.4 to 36°C for 4 or 8 h. For each 250 ml of cells (10^7 cells/ml), semi-intact cells were prepared as follows. Cells were collected by centrifugation (700 *g*, 7 min, RT), resuspended in 25 ml 100 mM Pipes, pH 9.4, 10 mM DTT, incubated with gentle agitation at 30°C for 10 min, and collected by centrifugation (1,000 *g*, 5 min, RT). Cells were resuspended in 6 ml YP, 0.2% glucose, 50 mM KPO_4, pH 7.5, 0.6 M sorbitol. 50 μl oxalyticase ([Enzogenetics] 40,000 U/ml stock in 50 mM KPO_4, pH 7.5) was added, and the suspension was incubated with gentle shaking 30°C for 15 min. Spheroplasting was monitored by light microscopy. Great care was taken not to overdigest cells to avoid lysis during the import reactions. Spheroplasts were collected by centrifugation at 1,000 *g* for 5 min at RT, resuspended with a plastic pipette in 40 ml YP, 1% glucose, 0.7 M sorbitol, and incubated with gentle shaking at 30°C for 20 min. Spheroplasts were collected by centrifugation (1,000 *g*, 5 min, RT) and washed twice at 4°C with cold permeabilization buffer (20 mM Pipes-KOH, pH 6.8, 150 mM K-Acetate, 2 mM Mg-Acetate, 0.4 M sorbitol. The final pellet was resuspended in 1 ml cold permeabilization buffer with 0.5 mM EGTA. 100-ml aliquots were placed in 1.5 ml microfuge tubes and frozen slowly above liquid N_2 and stored at −80°C. For import experiments, spheroplasts were thawed at RT, mixed with 0.9 ml of cold buffer A, washed twice with 1 ml buffer A, and resuspended in 0.4 ml of buffer A. Cells kept on ice were competent for import without loss of activity for at least 1 h.

Electron Microscopy

Samples containing import-competent permeabilized spheroplasts were fixed in 1.1% glutaraldehyde, 1.1% paraformaldehyde, 0.25% tannic acid, 0.1 M Na-cacodylate, pH 7.2, 150 mM K-Acetate, 5 mM Mg-Acetate, 0.4 M sorbitol for 36 h, postfixed with 1% reduced Osmium, stained with uranyl acetate, dehydrated and embedded in Spurr media. Sections were stained with lead citrate and viewed with a JOEL 100C TEM.

Import Assay

Import reactions were performed in buffer A and contained 5 × 10^7 cells/ml, 5 μg/ml HSA-conjugate, 1 mM ATP (Sigma Chem. Co., St. Louis, MO), 10 mM creatine phosphate (Boehringer Mannheim Corp., Indianapolis, IN), 0.1 mg/ml creatine kinase (Boehringer Mannheim Corp.), and 4 mg/ml cytosolic proteins. Reactions were incubated for 10 min at 30°C or 15 min at 23°C. The cells were mixed with 4',6'-diamidino-2-phenylindole (DAPI, final concentration 0.25 μg/ml) and analyzed by fluorescence microscopy at 1,000 × magnification with a Zeiss Axioscope equipped with a Plan Neofluor 100 × objective lens. When ATPγS was added, cells and cytosol were preincubated with adenosine 5'-O-(3-thiotriphosphate) (ATPγS, Sigma Chem. Co.) at 1 mM for 5 min before addition of the ATP-regenerating mix and HSA-peptide conjugate. For preincubation with antibodies, cells were incubated in buffer A with an IgG fraction

of anti-Nspl (Osborne et al., manuscript submitted for publication) or control IgG at 0.5 mg/ml and incubated for 10 min at 23°C. Cells were collected by centrifugation, washed once, and resuspended in buffer A. The effects of the various treatments and mutations (see below) were quantitated by counting ~200–300 spheroplasts in each experiment. Cells were scored as permeable if the nucleus was stained with DAPI and there was no obvious cell wall present.

For viewing by confocal microscopy, a BioRad MRC 600 confocal imaging device was used to process images from a Nikon microscope. Data were collected using a four scan average (Kalman filters). The intensity of signals was digitally enhanced using the contrast stretch function.

Preparation of Cytosol

Cells were grown in YPD at 30°C to a density of 5×10^7 cells/ml, collected by centrifugation at 2,000 *g* for 10 min, resuspended in 1/10 volume buffer A, centrifuged at 1,000 *g* for 5 min, resuspended in 1/10 volume buffer A, recentrifuged, and resuspended in 1/75 volume buffer A containing 1 mM DTT, 0.5 mM PMSF, 3 μg/ml leupeptin, aprotinin, chymostatin, and pepstatin A (all from Sigma Chem. Co.). Acid washed 0.5-mm glass beads were added at 1/3 the volume of the cell suspension, the mixture was transferred to an ice-cold bead beater, and subjected to 10 30-s pulses with 2 min intervals. The resulting lysate was centrifuged at 30,000 *g* for 15 min at 4°C and the supernatant further centrifuged at 100,000 *g* for 1 h and aliquoted and frozen in liquid N_2. The final protein concentration was 25–35 mg/ml.

For NEM treatment, cytosol was incubated with 3 mM *N*-ethylmaleimide (Calbiochem) for 5 min at 23°C. DTT was added to a final concentration of 4.5 mM and the mixture was incubated further for 5 min. For mock-treatment, NEM and DTT were added together followed by an incubation for 10 min.

Cytosol from HeLa cells was prepared as follows. Cells (5×10^6 cells/ml) were grown in suspension in SMEM with 10% calf serum, collected by centrifugation, washed twice with PBS, resuspended in 1/500 volume of 10 mM K-Acetate, 1.5 mM Mg-Acetate, 10 mM Pipes pH 6.8. After 10 min incubation on ice, cells were dounce homogenized and centrifuged as described for preparation of yeast cytosol.

Results

Semi-Intact Yeast Cells Import Proteins into the Nucleus

After conversion to spheroplasts, yeast cells can be made permeable to macromolecules by slow freeze-thawing. Using this approach, we introduced fluorescently labeled nuclear proteins into semi-intact cells and reconstituted their transport into the yeast nucleus. To accomplish this, we followed the original method of Baker et al. (1988) to isolate semi-intact yeast cells (see Materials and Methods). We chose to use diploid cells because of the slightly larger size of their nuclei. After freeze-thaw, the integrity of the nuclear envelope was examined in several ways. Cells were observed by electron microscopy (Fig. 1). A double membrane surrounding the nucleus as well as intact mitochondria and vacuoles were observed. However, holes were observed in the plasma membrane.

Macromolecules could gain access to the inside of the cells as evidenced by binding of antibodies against the yeast nucleoporin Nspl to the surface of the nucleus (Fig. 2, *A* and *B*). Semi-intact cells were incubated with anti-Nspl antibodies (Osborne, M., and G. Schlenstedt, unpublished results) followed by fluorescently tagged secondary antibodies and then viewed directly with the fluorescence microscope. Fluorescent punctate rings around the DAPI-stained nuclei (Fig. 2, *C* and *D*) were observed. This is the same fluorescence pattern seen with anti-Nspl antibodies in fixed intact yeast cells (Nehrbass et al., 1990; Stochaj et al., 1991).

On the other hand, antibodies against intranuclear proteins such as histone H2B or Npl3 (Bossie et al., 1992) did not bind to nuclei of semi-intact cells, consistent with the nuclear envelope providing a barrier to the nuclear interior (data not shown). When cells were treated with the detergent Triton X-100, nuclear staining with anti-histone antibody was observed. The staining patterns with anti-Nspl and anti-histone antibodies were the same when the cells were preincubated with cytosolic proteins (see below).

Not all cells were permeabilized by the freeze-thaw treatment. Typical examples are shown in Fig. 2. By Nomarski optics, some cells appeared to have retained the cell wall (Fig. 2, *E* and *F*). In fact, these same cells did not bind DAPI or anti-Nspl antibodies (compare *C* and *D* with *A* and *B* in Fig. 2). Based on these criteria (absence of DAPI staining of the nuclei and presence of a visible cell wall), in a typical experiment ~15–30% of the cells remained impermeable.

For an import substrate, we used the well-characterized conjugate of the SV40 large T-antigen NLS coupled to HSA

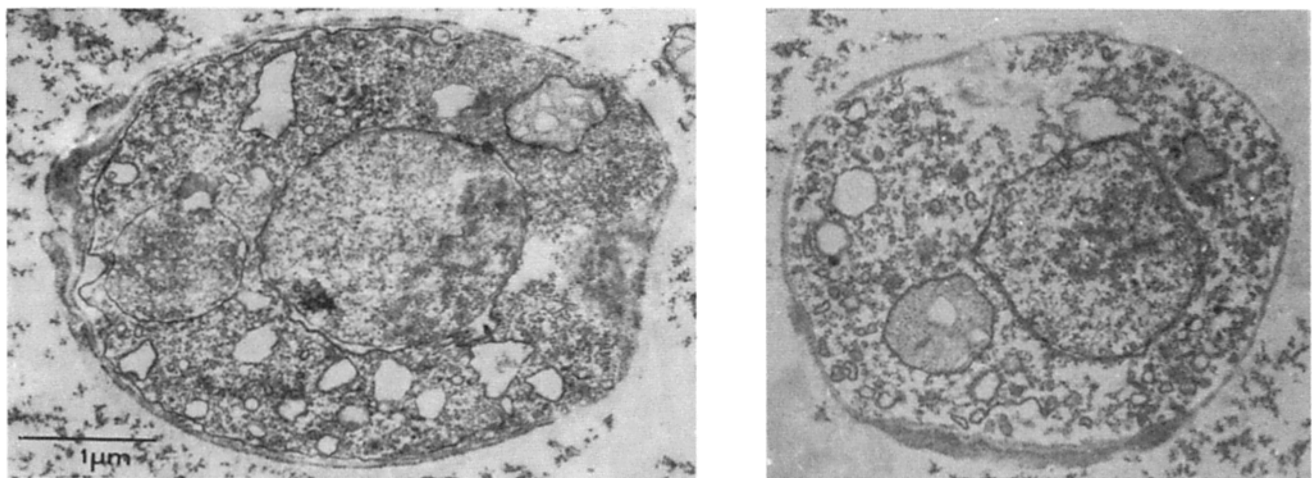
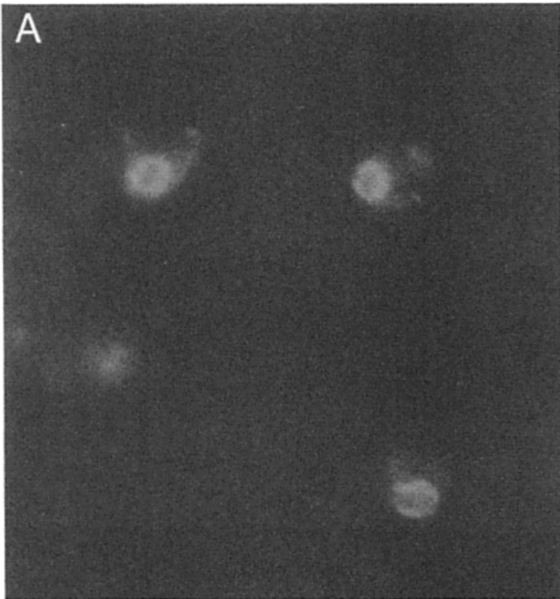


Figure 1. Ultrastructure of semi-intact yeast cells. Permeabilized cells were fixed, embedded, and stained for electron microscopy as described in Materials and Methods. Note the presence of intact double membranes around the nucleus, the partially removed cell wall, and the perforated plasma membrane.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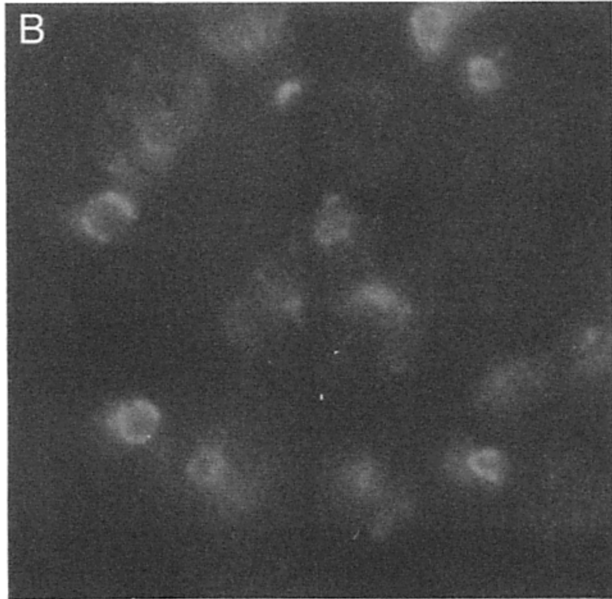
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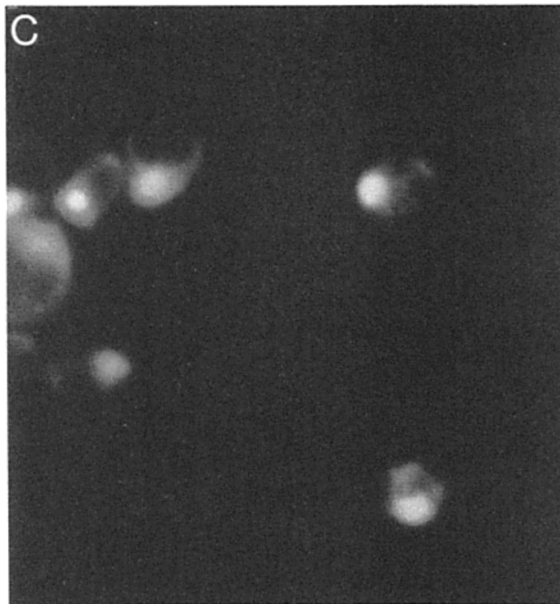
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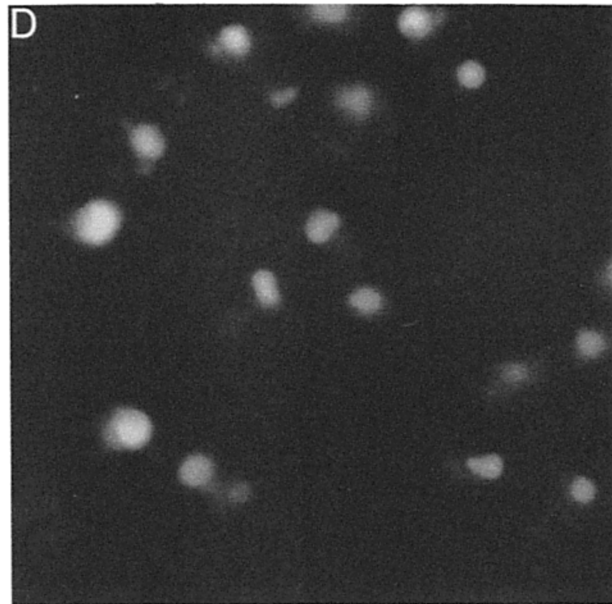
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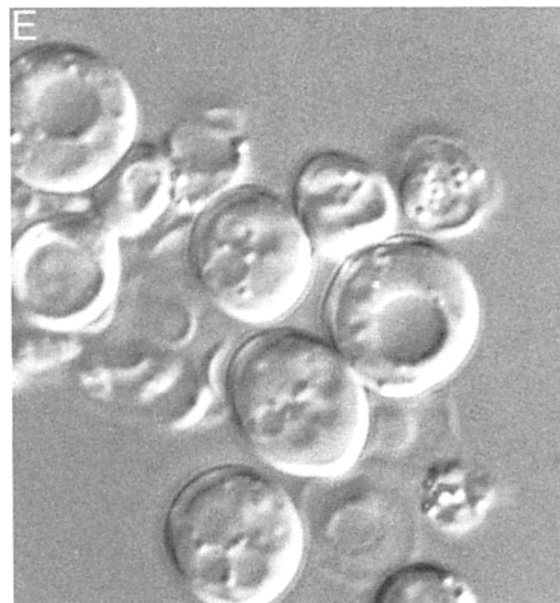
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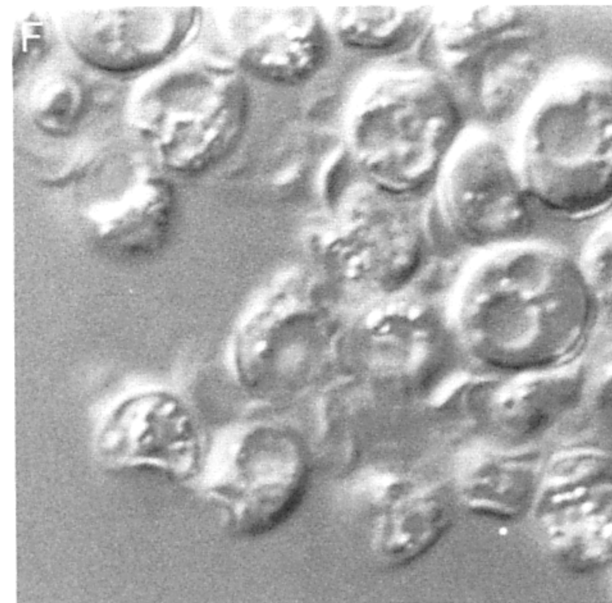
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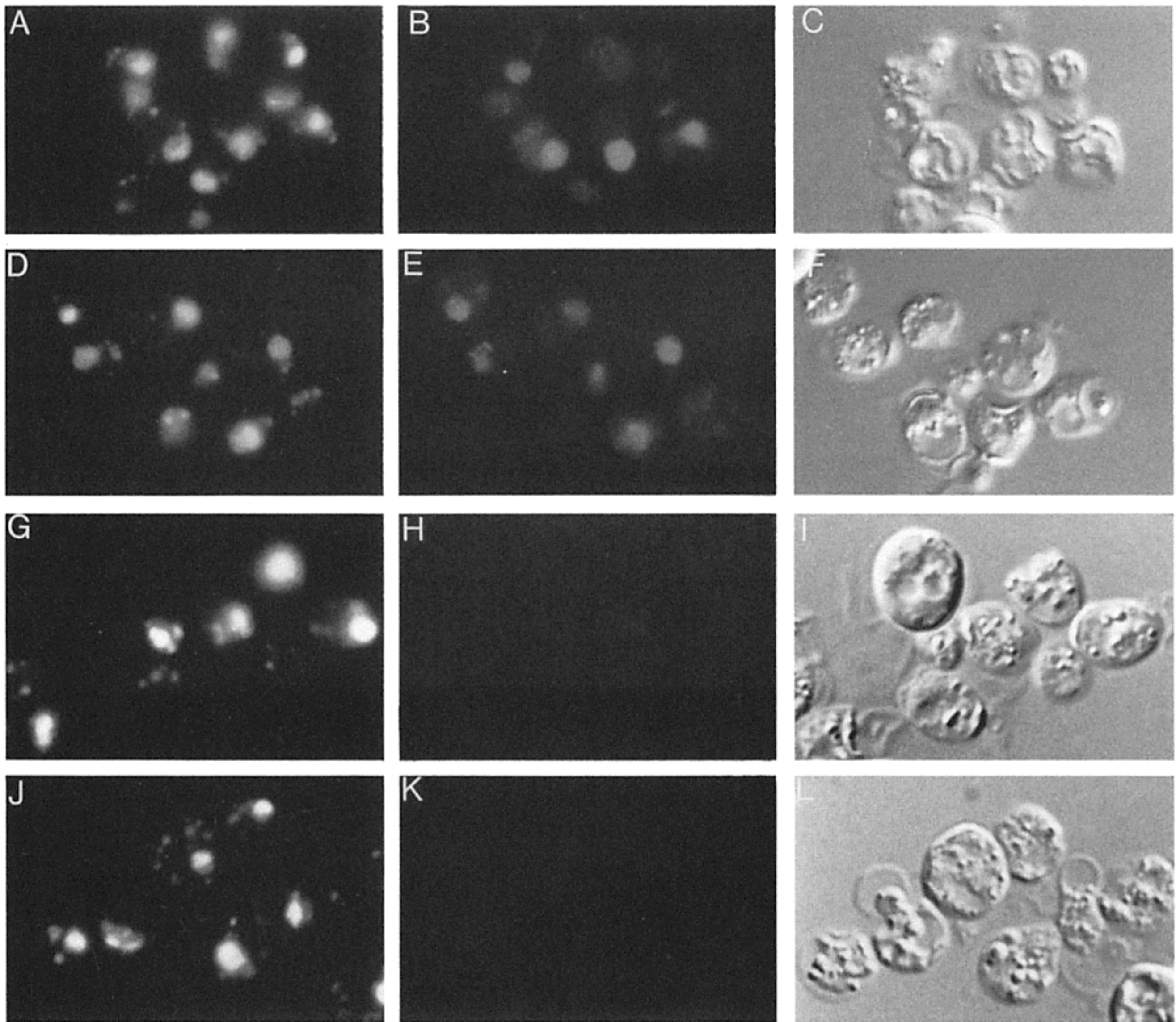

Figure 3. Nuclear protein association is specific in semi-intact yeast cells. Semi-intact cells were incubated with cytosol, ATP, and rhodamine-labeled SV40 NLS-HSA (*A–F*), rhodamine-labeled HSA conjugated with mutant SV40 NLSs (*G–I*), or rhodamine-labeled HSA (*J–L*) as described in Materials and Methods. After the import reaction, cells were viewed by fluorescence microscopy. HSA conjugates (*B, E, H,* and *K*), DAPI (*A, D, G,* and *J*), and Nomarski optics (*C, F, I,* and *L*).

(Goldfarb et al., 1986; Silver et al., 1989; Newmeyer and Wilson, 1991). In addition, the substrate was labeled with the fluorochrome lissamine rhodamine. After incubation of semi-intact cells with fluorescent NLS-HSA, yeast cytosol, and an ATP-regenerating system, association of the substrate within the nuclei was detected by fluorescence microscopy. The rhodamine fluorescence (Fig. 3, *B* and *E*) corresponded with the DAPI-stained nuclei (Fig. 3, *A* and *D*). On the other hand, when rhodamine-labeled HSA without a conjugated peptide (Fig. 3, *J–L*) or HSA conjugated with a mutated form of the NLS (Fig. 3, *G–I*) were used as substrate under identical conditions, no nuclear association was observed. For a typical import experiment, ~40% of the cells imported the wild-type NLS-HSA (Fig. 7 *A*).

Nuclear protein import has been observed to occur by a two step process: NLS-dependent binding followed by ATP- and cytosol-dependent translocation. We sought to test whether the same was true for semi-intact yeast cells. Without the addition of cytosol and ATP, only binding at the nuclear envelope occurred. Semi-intact cells were incubated with only rhodamine-labeled NLS-HSA and the reaction was observed by both epifluorescence microscopy (Fig. 4, *A–B*)

Figure 2. Visualization of the nuclear envelope with anti-Nspl antibodies. Semi-intact cells were treated with anti-Nspl antibodies followed by FITC-labeled anti-rabbit IgG and viewed by epifluorescence microscopy (*A* and *B*). *C* and *D* are the same cells stained with DAPI to visualize the DNA and *E* and *F* are the same cells viewed by Nomarski optics.

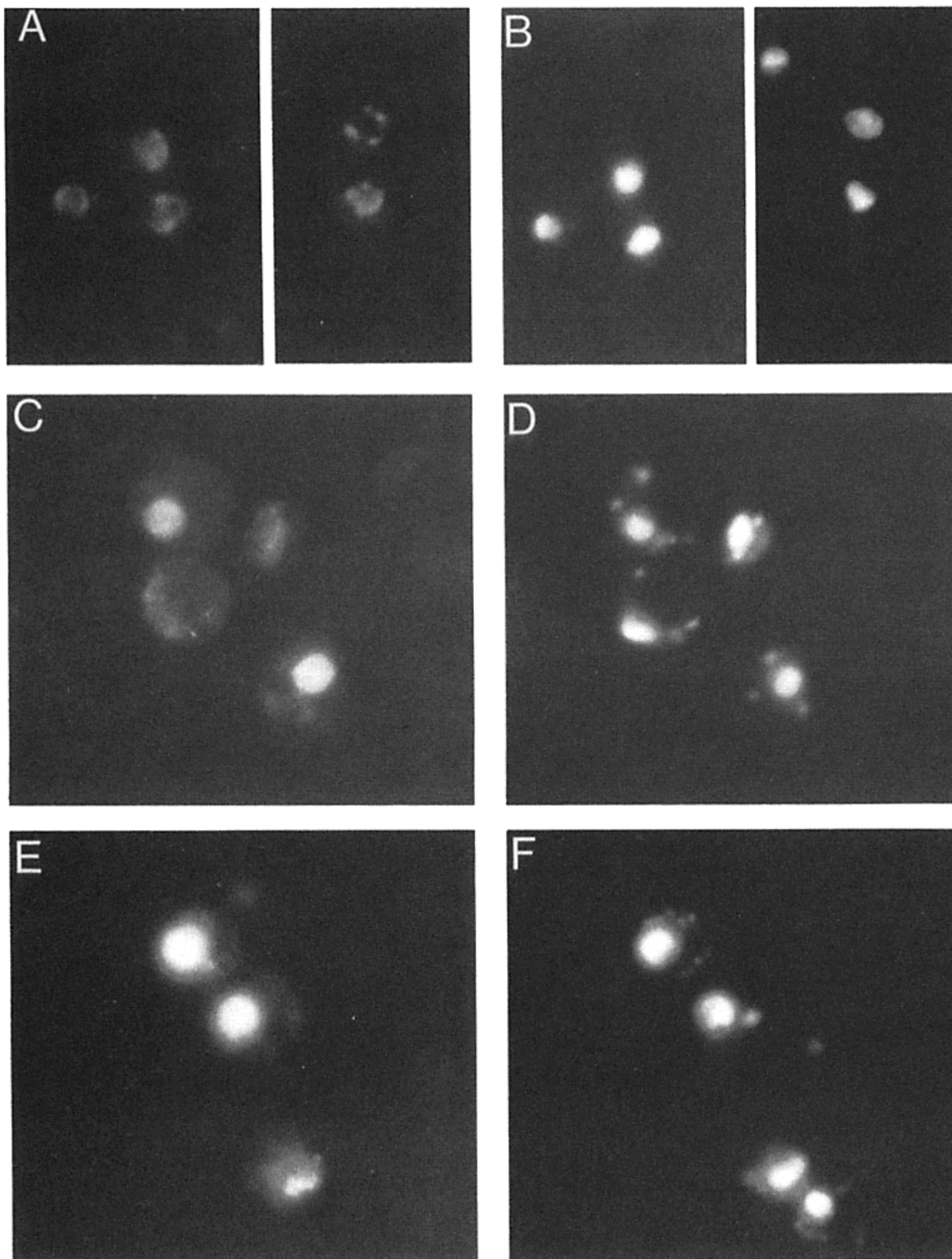

Figure 4. Nuclear import in semi-intact yeast cells requires cytosol. Semi-intact cells were incubated with rhodamine-labeled SV40 NLS-HSA without cytosol and ATP (*A* and *B*), in the presence of cytosol prepared from yeast strain ABYS1 and ATP (*C* and *D*), or in the presence of cytosol prepared from HeLa cells and ATP (*E* and *F*). NSL-HSA (*A*, *C*, and *E*); DAPI (*B*, *D*, and *F*).

and confocal microscopy (Fig. 5 *C*). The observed fluorescence was concentrated at the rim of the nuclei. In a typical experiment, 70–80% of the semi-intact cells displayed binding of NLS-HSA at the nuclear rim with no import into the nucleus when ATP and cytosol were absent (Fig. 7 *A*).

To confirm that binding of the substrate was occurring at the nuclear surface, cells were probed after incubation with NLS-HSA with anti-Nspl antibodies followed by FITC-labeled secondary antibodies (Fig. 5 *A*). When viewed by confocal microscopy, the fluorescence signals for both the substrate and Nspl were, for the most part, coincident (Fig. 5 *E*). To further confirm that the substrate accumulates in the nuclear interior when cytosol and ATP were present, cells were similarly probed with anti-Nspl after an import reaction (Fig. 5 *B*). When viewed by confocal microscopy, the fluorescence from the rhodamine-labeled NLS-HSA was mostly contained within a region defined by fluorescence with anti-Nspl antibodies (Fig. 5, *D* and *F*). Finally, we observed that NLS-HSA bound to the nuclear surface could be "chased" into the nucleus (Table I and Fig. 6). Taken together, these results indicate that nuclear protein import can be faithfully reconstituted with semi-intact yeast cells.

Factors Important for Nuclear Transport

Import of NLS-HSA into nuclei of semi-intact cells required addition of cytosol and ATP. Heating the cytosol for 5 min at 100°C rendered it inactive to support nuclear protein import. Only binding of NLS-HSA to the nuclear periphery was observed with heat-treated cytosol and ATP present. On the other hand, cytosol subjected to prolonged dialysis remained active in the import reaction.

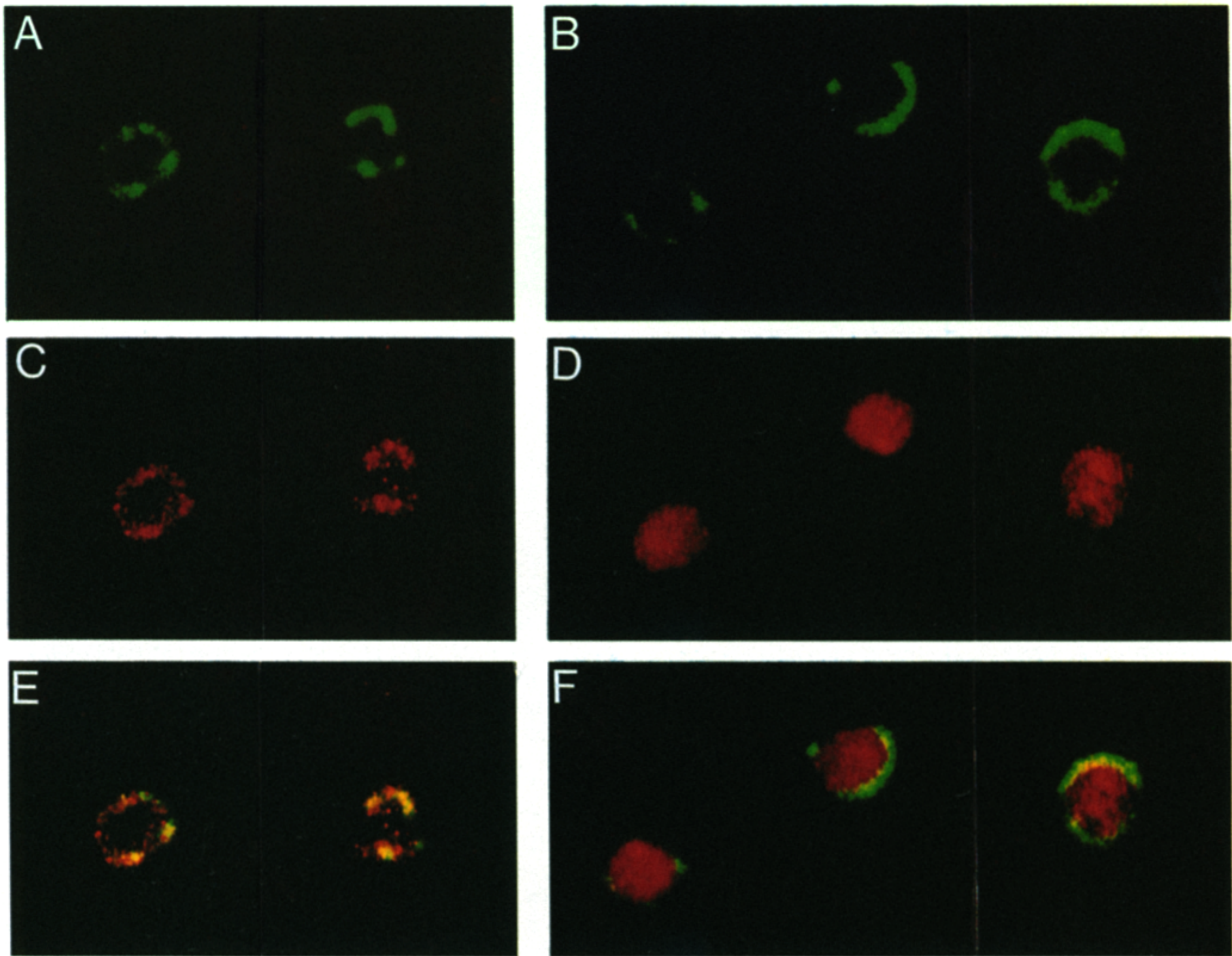

Figure 5. Localization of NLS-HSA in binding and import reactions. Semi-intact cells were incubated with rhodamine conjugated NLS-HSA in the absence of cytosol and ATP (*A*, *C*, and *E*) or in the presence of cytosol and an ATP-regenerating system (*B*, *D*, and *F*) as described in Materials and Methods. After the reactions, cells were incubated with anti-Nspl antibodies followed by FITC-conjugated anti-rabbit antibodies to visualize the nuclear envelope and nuclear pore complexes. Cells were viewed by confocal microscopy as described in Materials and Methods. *A* and *B* (*green*) visualize the anti-Nspl staining. *C* and *D* (*red*) visualize the rhodamine-labeled NLS-HSA and *E* and *F* are the merge of the two images showing colocalization as yellow.

To further investigate the role of ATP in the import reaction, semi-intact cells and cytosol were preincubated with the non-hydrolyzable analog ATPγS. Upon subsequent addition of ATP, creatine phosphate, creatine kinase, and substrate, nuclear surface binding and no import was observed (Fig. 8, *A* and *B*; Fig. 7 *A*), indicating a requirement for hydrolyzable ATP in the import reaction.

Table I. Coupling of NLS-HSA Binding to Nuclear Import

	No signal	Binding	Import
Reaction 1	19.5%	80.5%	0%
Reaction 2	16%	40%	44%

A binding reaction containing wild-type semi-intact cells and labeled NLS-HSA (see Materials and Methods) was incubated for 10 min at 30°C. An aliquot (*Reaction 1*) was removed and scored by fluorescence microscopy as described in Fig. 7. The remainder of the reaction received cytosol, ATP, and the ATP-regenerating system (*Reaction 2*), and was scored for the percentage of spheroplasts performing binding or import after further incubation for 10 min at 30°C.

We investigated whether the cytosol contains a protein(s) which is sensitive to cysteine alkylation. Yeast cytosol was treated with NEM followed by addition of DTT to inactivate the NEM or was mock treated. Semi-intact cells combined with the NEM-treated cytosol showed no import but only binding at the nuclear envelope (Fig. 8, *E* and *F*; Fig. 7 *A*). Semi-intact cells mixed with mock-treated cytosol showed the same amount of import as was observed in untreated cells (Fig. 8, *C* and *D*; Fig. 7 *A*). Similar NEM treatment of the semi-intact cells had no effect on their ability to carry out import.

We tested whether cytosol from other organisms could support import into yeast nuclei. Cytosol prepared from HeLa cells when used at a concentration of 0.8 mg/ml could support import of NLS-HSA into yeast nuclei (Fig. 4, *E* and *F*). Interestingly, the specific activity of HeLa cytosol was higher than for yeast (Fig. 4, *C* and *D*).

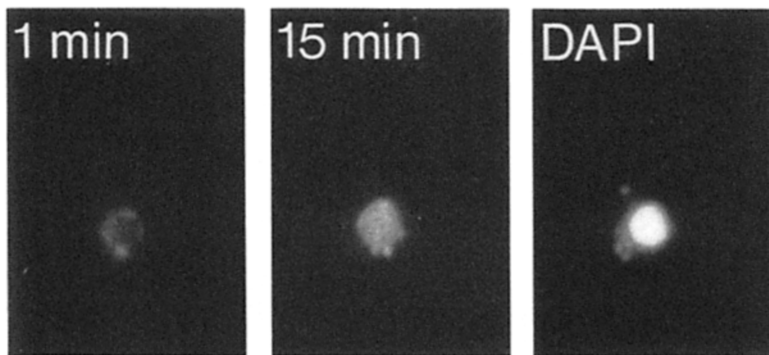

Figure 6. The bound form of NLS-HSA constitutes an import intermediate. An import reaction containing cytosol (4 mg/ml) was incubated for 1 h at 4°C. After addition of DAPI, semi-intact cells were mounted on a slide on ice. A photograph of a selected cell was taken 1 min after transfer of the slide to the fluorescence microscope and after leaving the slide for 15 min on the microscope at room temperature. The localization of labeled NLS-HSA (*1 min* and *15 min*) as well as DAPI staining is shown.

Taken together, these results show that yeast cells have the same requirements seen for nuclear protein transport in *Xenopus*, mammalian, and *Drosophila* cells. The reaction requires ATP and cytosol, and is sensitive to NEM. Moreover, the cytosol-supplied import factor(s) is/are conserved between species since cytosol from human cells can support import into yeast nuclei.

A Role for Nucleoporins in Nuclear Transport in Semi-Intact Cells

Several nucleoporins have been described for yeast (Nehrbass et al., 1990; Davis and Fink, 1990; Wente et al., 1992; Wimmer et al., 1992; Loeb et al., 1993). To test the role of Nspl in nuclear transport, we have generated polyclonal antibodies against Nspl (Osborne, M., G. Schlenstedt, and P. A. Silver, manuscript submitted for publication). As shown in Fig. 2, *A* and *B*, this antibody binds to the nuclear envelope. Preincubation of semi-intact cells with anti-Nspl IgG inhibited import of NLS-HSA (Fig. 9, *C* and *D*) whereas cells treated with the same concentration of control IgG displayed normal import (Fig. 9, *A* and *B*). However, we could still detect binding of NLS-HSA to the nuclear envelope in the presence of anti-Nspl antibodies indicating that the antibody inhibits import but not binding to the nuclear surface.

Mutations have been introduced into *NSPl* that render cells temperature-sensitive for growth (Nehrbass et al., 1990). After incubation at 36°C, *nspl^{ts}* cells failed to correctly localize NLS-containing proteins to the nucleus in vivo (Nehrbass, U., E. Faber, F. Dihlmann, W. Herth, and E. Hurt, manuscript submitted for publication). To test *nspl* cells in vitro, we prepared semi-intact cells from *nspl* cells grown at the permissive temperature of 23°C or from cells shifted for 4 h to the non-permissive temperature of 36°C. Cells grown at 23°C showed reduced but still significant levels of import of NLS-HSA in the in vitro assay (Fig. 7 *C*). On the other hand, no import of NLS-HSA was observed when *nspl* semi-intact cells prepared after shifting to the non-permissive temperature were combined with cytosol from wild-type cells (Fig. 10, *D–F*; Fig. 7 *C*). Binding to the nuclear surface still occurred but was partially defective since only 30% of the cells showed a reduced amount of binding of NLS-HSA per nucleus. Addition of Nspl purified from an overproducing *E. coli* strain (Osborne, M., G. Schlenstedt, and P. A. Silver, manuscript in preparation) did not restore import to *nspl* mutant semi-intact cells. Cytosol prepared from *nspl* cells shifted for 4 h to the non-permissive temperature of 36°C still supported import into semi-intact wild-type cells (Table II).

Temperature-sensitive *nsp49* mutants are also defective in vivo for nuclear protein localization (Doye, V., unpublished results). Semi-intact cells were prepared from *nsp49* mutants grown at either RT or after being shifted for 8 h to 36°C. For semi-intact cells prepared from a culture shifted to the non-permissive temperature, no import or binding of NLS-HSA was observed (Fig. 10, *G–I*; Fig. 7 *C*). There was, however, some diffuse binding throughout the cell cytoplasm. As with *nspl*, cytosol prepared from *nsp49* cells shifted for 8 h to the non-permissive temperature of 36°C still supported import into semi-intact wild-type cells (Table II).

As a control for the temperature-sensitive mutants, we tested semi-intact cells prepared from a temperature-sensitive mutant in the *NOPl* gene (Schimmang et al., 1989). Nopl is a nucleolar protein important for maturation of ribosomes (Tollervey et al., 1993). *Nopl ts* mutants cease growth after 8 h at 36°C, but no effect on nuclear protein import is observed in vivo (Nehrbass et al., 1990). We found the same to be true for semi-intact cells prepared from *nopl* mutants shifted to 36°C. These cells showed almost normal import of NLS-HSA when provided with wild-type cytosol and ATP (Fig. 10, *A–C*; Fig. 7 *B*). Wild-type cells grown at 36°C also showed no defect in nuclear import when semi-intact cells were prepared (Fig. 7 *B*).

Npl3 Mutants Are Blocked in Import and Binding

We have reported the isolation and characterization of *npl3* mutants of yeast that are blocked in import of nuclear proteins (Bossie et al., 1992). *NPL3* encodes a nuclear protein with similarity to a family of RNA-binding proteins that shuttle between the cytosol and the nucleus. *npl3-1* mutants are temperature-sensitive for growth and for nuclear protein import and mRNA export. (Bossie, M., L. Gorsch, C. Cole, and P. Silver, unpublished results). We prepared semi-intact cells from *npl3-1* cells shifted for 4 h to 36°C or grown at permissive room temperature. When mixed with wild-type cytosol and ATP, no import or binding of NLS-HSA was observed in semi-intact cells prepared from cells grown at the non-permissive temperature (Fig. 10, *J–L*, Fig. 7 *B*). Instead, we observed a diffuse binding throughout the cell cytoplasm. Semi-intact cells prepared from *npl3-1* mutants grown at permissive temperature showed wild-type levels of import of NLS-HSA (Fig. 7 *B*). Cytosol prepared from *npl3*

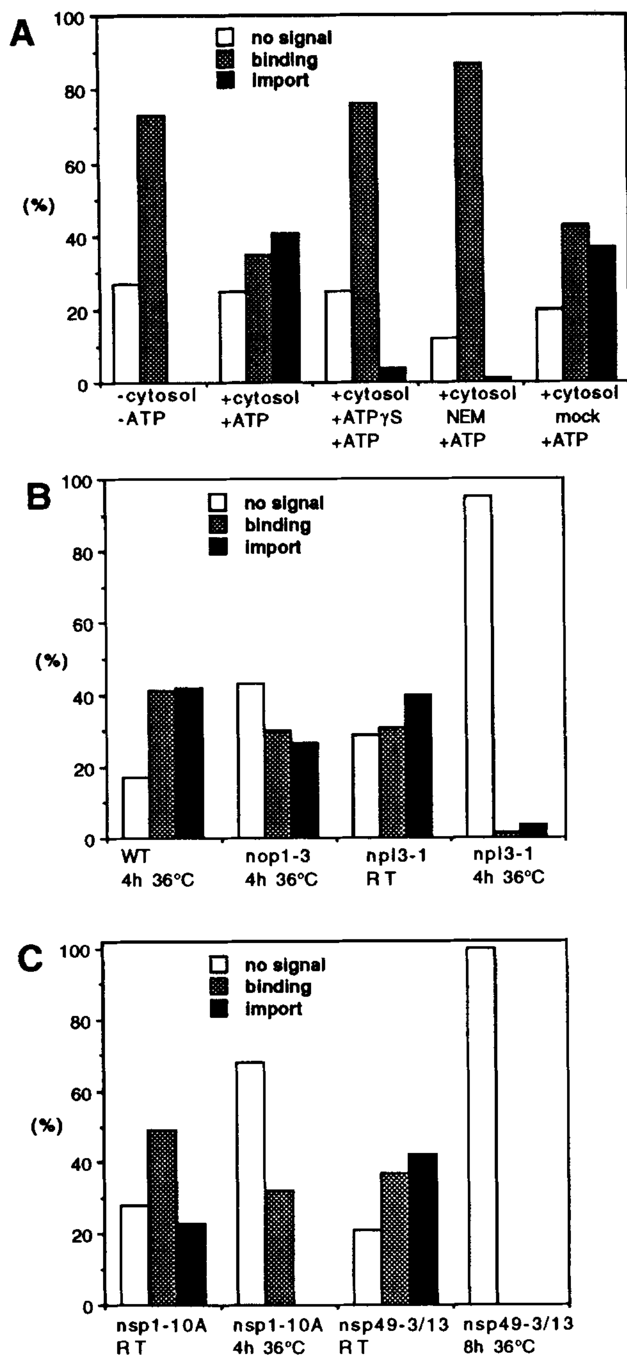

Figure 7. Quantitation of effects of various treatments and mutations on nuclear transport in semi-intact yeast cells. For each experiment, ~300 spheroplasts were scored as indicated where accumulation of NLS-HSA within the nucleus was defined as "import," punctate ring staining was defined as "binding," and neither was defined as "no signal." (*A*) The effects of cytosol, ATPγS, and NEM treatment of cytosol on nuclear uptake of rhodamine-labeled NLS-HSA into wild-type semi-intact cells. (*B*) Import assay with semi-intact cells prepared from either wild-type, *nopl-3,* or *npl3-1* mutants. (*C*) Import assay with semi-intact cells from *nspl-10A* and *nsp49-3/13* mutants.

mutant cells grown at the non-permissive temperature of 36°C was capable of supporting import when mixed with wild-type semi-intact cells (Table II).

Discussion

Proper nuclear protein import has been reconstituted using semi-intact yeast cells. The uptake of fluorescently tagged protein into nuclei of wild-type semi-intact yeast cells depends on a functional NLS, ATP, and the presence of cytosol. In the absence of cytosol and ATP, only NLS-dependent binding at the nuclear envelope is observed. The cytosol contains at least one NEM-sensitive component that is necessary for import. Overall, our results with semi-intact yeast cells are similar to those found with mammalian and *Drosophila* semi-intact tissue culture cells and with *Xenopus* oocyte extracts. That is, nuclear protein transport can be divided into two steps; ATP-independent binding at the nuclear envelope followed by cytosol and ATP-dependent import across the nuclear envelope.

Using this reconstituted system, it is now possible to investigate yeast mutants and distinguish between a binding and import defect caused by a given mutation. Furthermore, this reaction can be used to determine whether a protein displays a defect at the level of the cytoplasm or the nucleus and whether isolated proteins or antibodies influence nuclear transport. For example, preincubation of semi-intact cells with antibodies against the yeast nucleoporin Nspl blocks protein import but not binding to the nuclear envelope. In addition, semi-intact cells prepared from cells mutated in *NSPl* are defective in nuclear protein import but some binding still occurs. Taken together, these results suggest that Nspl is directly involved in the import reaction and not with binding to the nuclear pore complex. Alternatively, Nspl could also be involved in binding but the temperature-sensitive mutation, as well as binding of the anti-Nspl antibody only eliminated the import function.

Mutations in the nucleoporin Nsp49 completely block binding of NLS-HSA to the nuclear envelope. Several interpretations are consistent with our observations. First, Nsp49 may bind NLSs directly. Second, Nsp49 may interact with factors important for binding NLSs. Third, mutation of Nsp49 may alter the structure of the pore complex. The observation that no import takes place when binding is blocked supports the two step model of nuclear protein transfer.

We have previously described mutations in *NPL3* that cause a temperature-sensitive defect in nuclear protein import (Bossie et al., 1992). We now show that this defect is caused by a block in the binding step. In agreement with that, we previously reported that NLS-binding proteins are dissociated from their normal nuclear location in *npl3* mutants (Stochaj et al., 1993). The results presented here indicate that the block in *npl3* cells is at the level of the nucleus since cytosol from *npl3* mutants can support productive import. *NPL3* encodes a nuclear protein with similarity to RNA-binding proteins that are proposed to shuttle in and out of the nucleus. One possible explanation for the binding defect in *npl3* mutants is that mutant Npl3 is physically jamming the import machinery. Another explanation is that mutant Npl3 could be titrating a cytosolic or a nuclear factor necessary for transport.

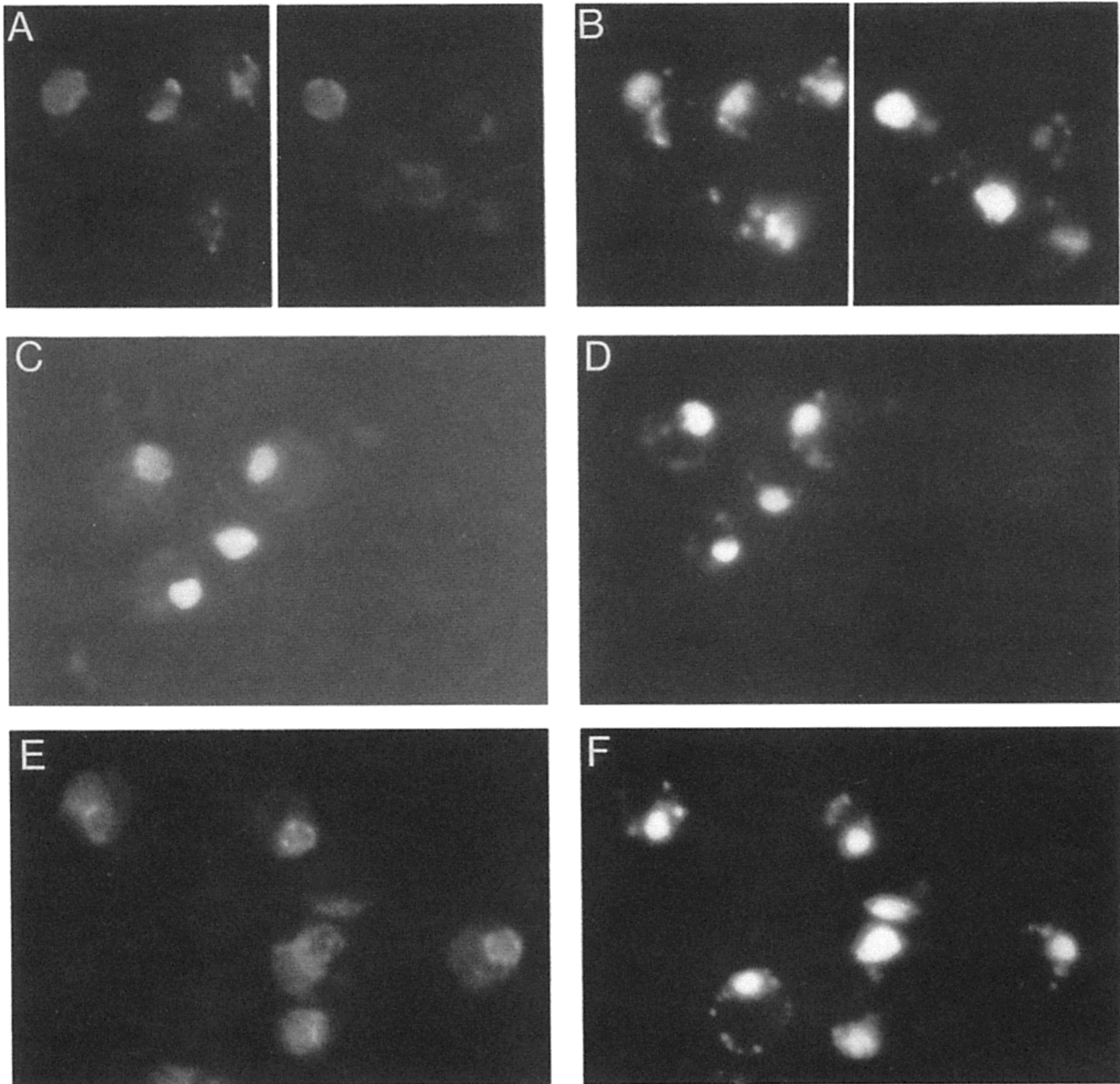

Figure 8. The role of ATP and effect of NEM-treated cytosol in nuclear transport in semi-intact yeast cells. Rhodamine-labeled NLS-HSA was incubated with wild-type semi-intact yeast cells and cytosol that had both been preincubated with ATPγS (*A* and *B*). Cells were incubated with ATP, the import substrate, and mock-treated (*C* and *D*) or NEM-treated cytosol (*E* and *F*) prepared as described in Materials and Methods. NLS-HSA was visualized by fluorescence microscopy (*A*, *C*, and *E*). The same cells were stained with DAPI (*B*, *D*, and *F*).

Previous reports (Kalinich and Douglas, 1989; Garcia-Bustos et al., 1991*b*) describe the uptake of SV40 large T-antigen, nucleoplasmin, and Mcml by isolated yeast nuclei. This was dependent on time, temperature, ATP, calcium, and the presence of a functional NLS but did not require addition of yeast cytosol. However, the imported proteins were synthesized in a reticulocyte lysate which was then added directly to the nuclei. It is possible that the reticulocyte lysate could provide necessary cytosolic import factors since it is known to contain active NLS-binding proteins (Adam et al., 1991). Also, protein import was monitored by inaccessibility to digestion by trypsin coupled to agarose beads and not visually demonstrating an intranuclear location as we now report.

We previously reported the specific association of SV40 NLS-containing fluorescent proteins with nuclei of semi-intact cells and with isolated yeast nuclei (Silver et al., 1989; Stochaj et al., 1991). This association was dependent on a functional NLS and is proposed to occur via interaction with specific NLS-binding proteins (Stochaj et al., 1991). Consistent with the results presented here, no cytoplasm was required to observe NLS-dependent binding to nuclei.

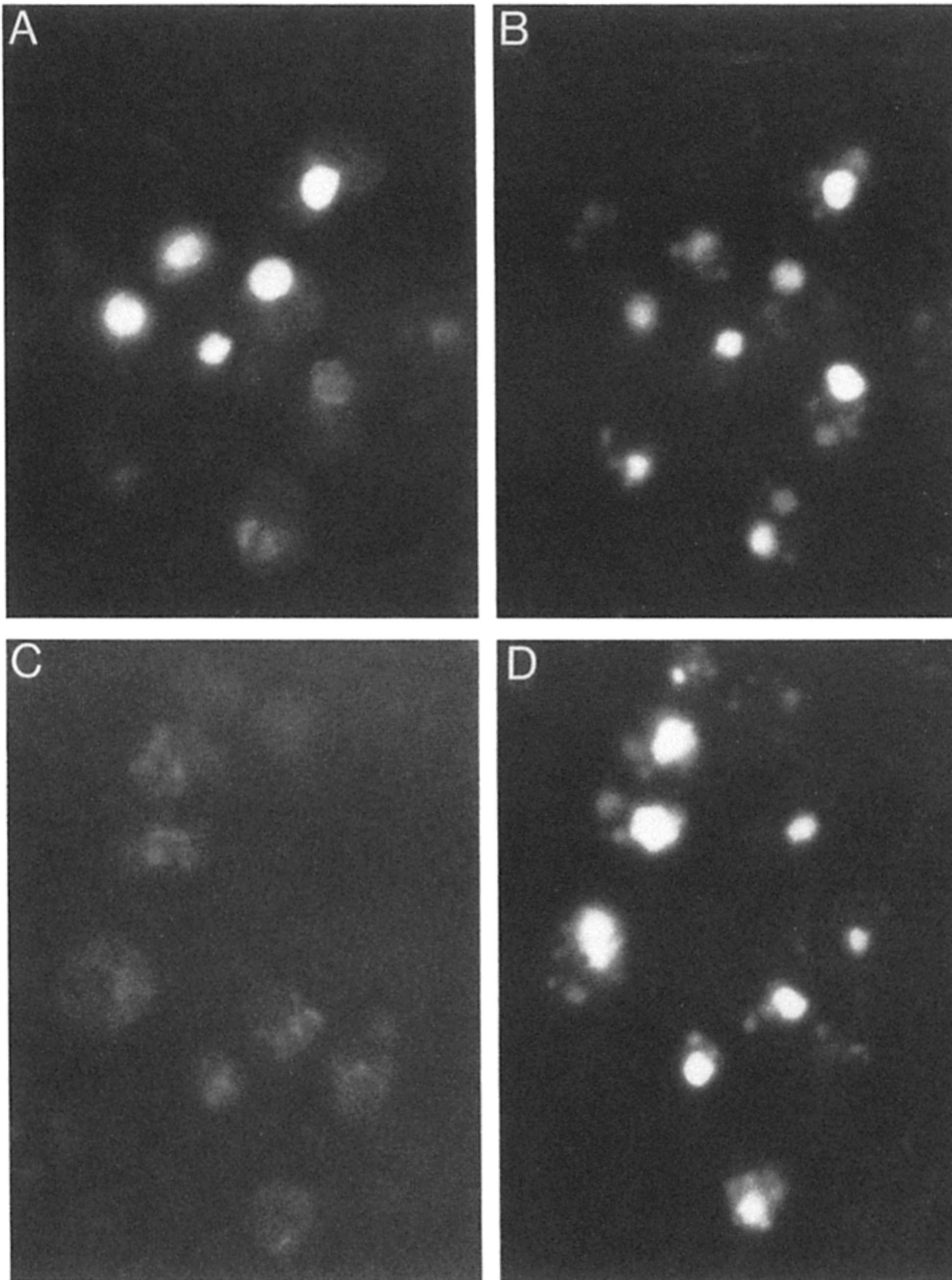

Figure 9. Anti-Nspl antibodies inhibit import but not binding. Wild-type semi-intact cells were preincubated with anti-beta-galactosidase IgG (*A* and *B*) or with anti-Nspl IgG (*C* and *D*), and then tested for import of rhodamine-labeled NLS-HSA (*A* and *C*). *B* and *D* are the same cells stained with DAPI.

Several proteins have been described in yeast that specifically bind NLS-containing proteins (Silver et al., 1989; Lee and Melese, 1989) and have been proposed to act as receptors for nuclear protein import. We have shown that one of these proteins, a phosphoprotein termed NBP70, is associated mostly with yeast nuclei (Stochaj et al., 1991). Nuclei missing NBP70 no longer display NLS-dependent protein binding. An immunocross-reactive phosphoprotein with similar biochemical characteristics exists in cells from other species (Stochaj and Silver, 1992). We, and others, have proposed that NLS-binding proteins, like NBP70, may recognize proteins in the cytoplasm and deliver them to the nuclear envelope. The binding of NLS-HSA that we observe in the experiments presented here is consistent with the idea that some of these proposed NLS-receptors reside at the nuclear envelope in yeast. This binding could reflect a pool of receptors bound to the pore. We do not detect any NBP70 in the cytosol preparation used in the in vitro reaction (data not shown). It could be that association of NBPs primarily with the nucleus is particular to yeast due to their closed mitosis.

The data presented here support the proposal that import is occurring at the nuclear pores. First, import, but not binding, was blocked by pretreatment with antibodies against Nspl, a known yeast nucleoporin. Second, semi-intact cells prepared from cells containing a temperature-sensitive *nspl*

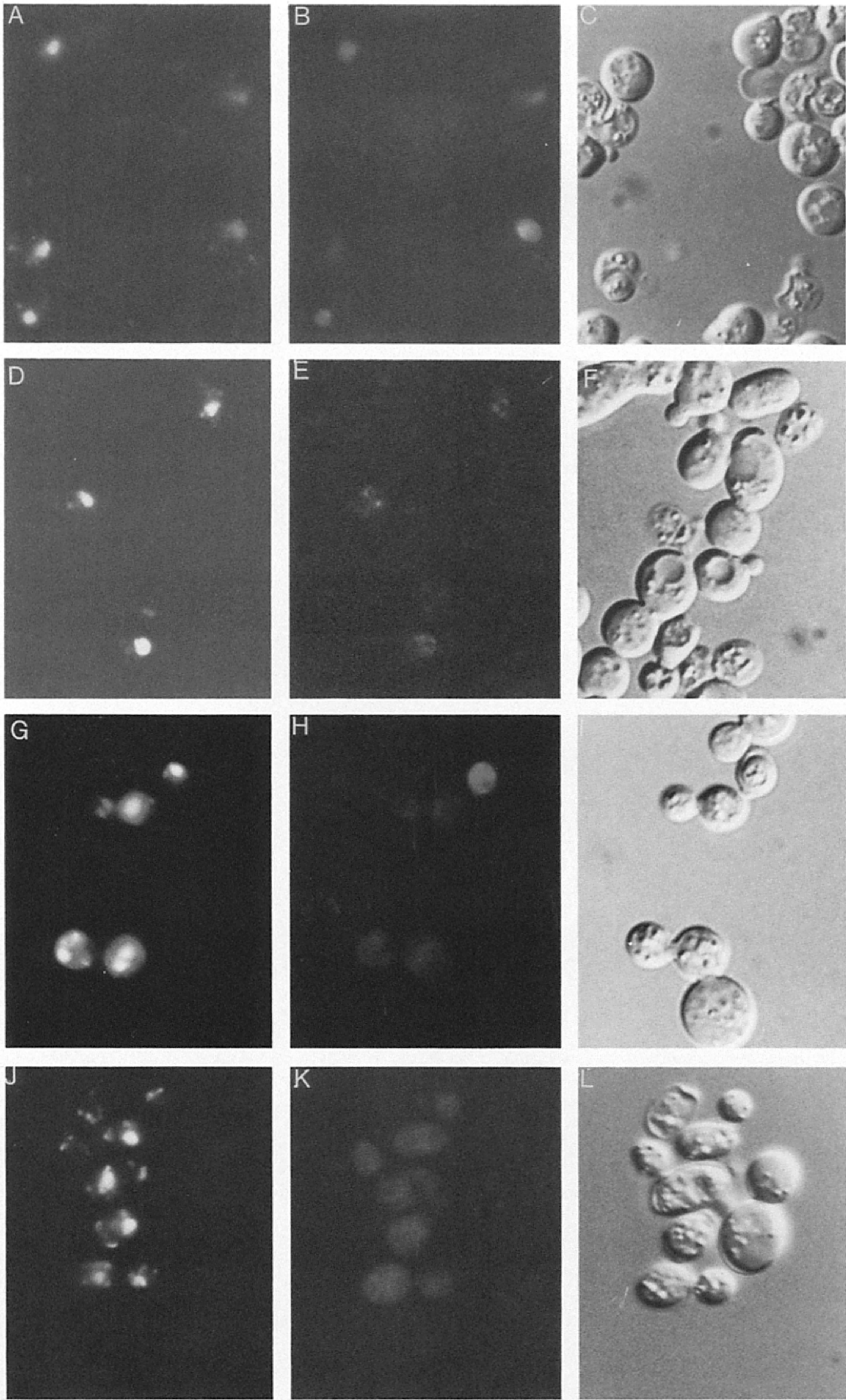
A
B
C
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Table II. The Import Defect Is Nuclear-associated in Temperature-Sensitive Mutants

Cells		Cytosol		Binding	Import
WT	(0)	WT	(4)	+	+
WT	(0)	*npl3-1*	(4)	+	+
WT	(0)	*nsp1-10A*	(4)	+	+
WT	(0)	*nsp49-3/13*	(8)	+	+
npl3-1	(4)	WT	(0)	−	−
npl3-1	(4)	*npl3-1*	(4)	−	−
nsp1-10A	(4)	WT	(0)	(+)*	−
nsp1-10A	(4)	*nsp1-10A*	(4)	(+)	−
nsp49-3/13	(8)	WT	(0)	−	−
nsp49-3/13	(8)	*nsp49-3/13*	(8)	−	−

Yeast cultures from wild-type (*WT*) or mutant strains were shifted to 36°C for the number of hours indicated in parentheses before preparation of semi-intact cells or cytosol. Import reactions (see Materials and Methods) containing cytosol in limiting amounts (final concentration 1 mg/ml) were incubated for 10 min at 30°C. Aliquots were scored by fluorescence microscopy for binding and import or labeled NLS-HSA.

* Binding in *nsp1* mutants was reduced compared to WT.

allele were also defective in import but not binding of NLS-HSA. Finally, we observed colocalization of the bound form of the import substrate and Nspl by confocal microscopy.

There is much evidence that the components of the nuclear protein import machinery are conserved. NLSs originally defined in mammalian cells function correctly in yeast (e.g., Nelson and Silver, 1989) and similar NLS-binding proteins have been defined in a number of different species (Stochaj and Silver, 1992). Also, yeast nuclear pore proteins have been identified by cross-reactivity with antibodies raised against mammalian nuclear pores (Davis and Fink, 1990; Wente et al., 1992; Loeb et al., 1993). In this study, we have shown that the cytosol from mammalian cells supports nuclear protein import in semi-intact yeast cells. Therefore, the cytosolic import factors are functionally conserved and the mammalian factors can complement the yeast factors in mixed biochemical assays.

The ease of the assay presented here and the availability of a number of additional mutants that affect nuclear transport should lead to progress in further understanding of nuclear transport. Future experiments will focus on attempts to rescue the transport defects observed in the various mutants.

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Figure 10. Effect of temperature-sensitive mutations on nuclear import. Semi-intact cells were prepared form *nopl* mutants (*A–C*), *nspl* mutants (*D–F*), *nsp49* mutants (*G–I*), and *npl3* mutants (*J–L*) and tested for nuclear import of NLS-HSA (*B, E, H,* and *K*) as described in Materials and Methods. *A, D, G,* and *J* are the same cells stained with DAPI and *C, F, I,* and *L* are the same cells viewed by Nomarski.

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