

# Nuclear Protein Import in Permeabilized Mammalian Cells Requires Soluble Cytoplasmic Factors

Stephen A. Adam, Rachel Sterne Marr, and Larry Gerace

Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037

**Abstract.** We have developed an in vitro system involving digitonin-permeabilized vertebrate cells to study biochemical events in the transport of macromolecules across the nuclear envelope. While treatment of cultured cells with digitonin permeabilizes the plasma membranes to macromolecules, the nuclear envelopes remain structurally intact and nuclei retain the ability to transport and accumulate proteins containing the SV40 large T antigen nuclear location sequence. Transport requires addition of exogenous cytosol to permeabilized cells, indicating the soluble cytoplasmic factor(s) required for nuclear import are released during digitonin treatment. In this reconstituted import system, a protein containing a nuclear location signal is rapidly accumulated in nuclei, where it reaches a 30-fold concentration compared to the surrounding medium within 30 min. Nuclear import is specific for a functional nuclear location sequence, re-

quires ATP and cytosol, and is temperature dependent. Furthermore, accumulation of the transport substrate within nuclei is completely inhibited by wheat germ agglutinin, which binds to nuclear pore complexes and inhibits transport in vivo. Together, these results indicate that the permeabilized cell system reproduces authentic nuclear protein import. In a preliminary biochemical dissection of the system, we observe that the sulfhydryl alkylating reagent *N*-ethylmaleimide inactivates both cytosolic factor(s) and also component(s) in the insoluble permeabilized cell fraction required for nuclear protein import. Because this permeabilized cell model is simple, efficient, and works effectively with cells and cytosol fractions prepared from a variety of different vertebrate sources, it will prove powerful for investigating the biochemical pathway of nuclear transport.

**T**RANSPORT of molecules between the cytoplasm and nucleus occurs through the nuclear pore complex, a large proteinaceous structure that spans the nuclear envelope (18). The pore complex has a complicated architectural organization and a mass of  $\sim 125 \times 10^6$  D, but only a small fraction of its proteins have been identified and characterized. These include an integral membrane glycoprotein called gp210 (19), and a group of peripheral membrane proteins containing O-linked *N*-acetylglucosamine (7, 21–23, 44). While detailed functional information has not been obtained on specific pore complex proteins, in recent years general features of transport across the pore complex have been delineated by a combination of structural and physiological approaches.

Cell microinjection studies have shown that the pore complex contains an aqueous channel of  $\sim 10$  nm diameter that allows nonselective passive diffusion of small molecules and metabolites across the nuclear envelope (40). Macromolecules larger than 30–40 kD cannot rapidly diffuse across this 10-nm channel, and appear to be transported between the cytoplasm and the nucleus by selective mediated mechanisms (11). In these cases, the pore complex channel can be ex-

panded, or gated, to allow rapid transport of particles up to  $\sim 30$  nm in diameter (8, 9). Transport of most proteins and RNA through the pore complex requires energy and is temperature dependent, and therefore is thought to reflect an active process. Individual pore complexes apparently can carry out both protein import and RNA export, and some steps of these two processes occur simultaneously (9). Thus, the pore complex appears to be an elaborate biochemical machine that can coordinate bidirectional molecular transport across the nuclear envelope.

Most available information on macromolecular transport across the pore complex has come from analysis of nuclear protein import. Transport of many proteins into the nucleus is directed by short amino acid sequences present in the proteins known as nuclear location sequences (NLSs'; 18). NLSs appear to specify interaction with the pore complex, since at reduced temperature or in the absence of ATP colloidal gold particles coated with proteins containing NLSs bind to the pore complex but are not transported into the nucleus (36, 41). These binding events may represent intermediates in the pathway of protein import that reflect interaction of NLSs with transport machinery of the pore complex. It has

Rachel Sterne Marr's present address is Merck, Sharp, and Dohme Research Laboratories, West Point, Pennsylvania 19486.

1. *Abbreviations used in this paper:* APC, allophycocyanin; NEM, *N*-ethylmaleimide; NLS, nuclear location sequence; WGA, wheat germ agglutinin.

been suggested that the NLS provides the signal for gating of the nuclear pore complex channel (2, 12, 38).

A prototypical NLS was described for the SV40 large T antigen that is necessary and sufficient to direct the T antigen from the cytoplasm to the nucleus (28). It comprises the sequence PK<sup>128</sup>KRRKV. Mutations in this sequence that replace the second lysine residue greatly diminish its ability to direct nuclear accumulation (25, 28). Most well-characterized NLSs of nuclear proteins contain a short stretch of basic residues, often flanked by a proline or glycine residue, similar to the NLS of the SV40 T antigen. NLSs can be artificially incorporated in the genes for large nonnuclear proteins and effectively direct nuclear import of the resulting proteins. In addition, synthetic peptides comprising NLS have the ability to direct nuclear import when chemically coupled to non-nuclear carrier proteins (20, 29).

Recently, two proteins that interact with an NLS with the specificity and high affinity expected for a transport receptor were identified by chemical crosslinking in both the nucleus and cytosol of rat liver (1). Other studies have identified a number of proteins that bind NLS in rat liver, human cells (32, 46), and yeast (31, 43). A role for any of these proteins in nuclear protein import has not been established by functional approaches.

Several systems have been described that reconstitute nuclear protein import in vitro to enable biochemical dissection of the transport machinery. The best-characterized system involves *Xenopus* egg extracts, which contain structural components of the nucleus in a disassembled state. Egg extracts can assemble intact nucleus-like structures around added DNA (33). Furthermore, when isolated rat liver nuclei (which are not structurally intact) are incubated with egg extracts, a significant proportion of the rat nuclei become sealed by incorporating *Xenopus* nuclear envelope components. These nuclei exclude nonnuclear proteins and selectively accumulate *Xenopus* nucleoplasmin or carrier proteins conjugated to synthetic peptides containing NLSs (36–39). Ultrastructural techniques demonstrated that this in vitro transport occurs through nuclear pore complexes. In addition, the lectin wheat germ agglutinin (WGA), which binds to nuclear pore complexes and inhibits nuclear protein import in intact cells (6, 45, 47), inhibits nuclear import with the cell-free system (14). Thus, this *Xenopus* system reproduces major features of nuclear protein import seen in vivo. Other systems to analyze nuclear import have been described using isolated nuclei from rat liver or yeast (24, 26, 34). While association of proteins with nuclei in these systems reflects some of the characteristics of in vivo protein transport, it has not yet been demonstrated that the nuclei are structurally intact and that protein association with nuclei involves transport through pore complexes.

All of the cell-free transport systems that have been described thus far have the limitation of utilizing nuclei that have been dissociated from other cellular structures. An intact nuclear envelope is essential to obtain transport-related nuclear accumulation of proteins, but it is difficult to isolate intact nuclei using mechanical homogenization of cells. This can be overcome by repair of the nuclear envelope as done with the *Xenopus* egg extract, but this results in mixing of heterologous nuclear envelope components and possible modification of pore complexes by *Xenopus* components, thereby complicating analysis of the transport machinery. To

study the interactions between the nucleus and the cytoplasm necessary for transport of molecules across the nuclear envelope, it would be desirable to have an in vitro system that preserves as much of the native architecture of the cell as possible, yet can be easily fractionated biochemically.

The results presented in this paper describe a permeabilized cell system for the study of nuclear protein import that satisfies these requirements. This assay is both rapid and simple relative to previously published protocols. The permeabilized cells efficiently and faithfully mimic nuclear protein transport in intact cells in terms of energy requirements and the effects of nuclear protein transport inhibitors. This system has the added advantage that the nuclear envelope remains intact throughout the procedure and that the basic higher-order structure of the cell is maintained. Using this system, we demonstrate that soluble cytoplasmic factor(s) are involved in transport of proteins from the cytoplasm to the nucleus.

## Materials and Methods

### Cell Culture

Human (HeLa) cells and normal rat kidney (NRK) cells were grown in DME (Gibco Laboratories, Grand Island, NY) containing 10% FBS (HyClone Laboratories, Logan, UT) and penicillin/streptomycin. Cultures were maintained in a humidified incubator with 5% CO<sub>2</sub> atmosphere. Suspension cultures of HeLa cells or a rat hepatoma cell line (HTC) were grown in Joklik's modified minimum essential medium with 5% FBS. Cells were removed from plastic dishes by trypsinization and replated on glass coverslips 24–48 h before use.

### Preparation of Fluorescent Conjugates

Synthetic peptides containing the SV40 large T antigen wild type (CGG-GPKKKRKVED) or a mutant transport-deficient (CGGGPKNKKRKVED) nuclear location signal were obtained from Multiple Peptide Systems (San Diego, CA). Before conjugation, the peptides were resuspended in 50 mM HEPES, pH 7.0, and reduced with 50 mM DTT. Reduced peptides were separated from the DTT by chromatography on Sephadex G-10. The peptides containing reduced amino-terminal cysteine residues were mixed at a 50-fold molar excess with the phycobiliprotein allophycocyanin (APC; Calbiochem-Behring Corp., San Diego, CA) that had previously been activated with a 20-fold molar excess of sulfo-SMCC (Pierce Chemical Co., Rockford, IL). After overnight incubation at 4°C the APC-peptide conjugates were separated from free peptides by desalting on Sephadex G-25. The number of peptides conjugated to the protein was estimated by mobility shift on SDS-polyacrylamide gels to be approximately four to eight peptides per APC molecule. The conjugates were then dialyzed against 10 mM HEPES, pH 7.3, 110 mM potassium acetate and stored at 4°C. The concentration of the allophycocyanin conjugates was determined by absorbance at 650 nm.

### Preparation of Cytosol Fractions

Exponentially growing cultures of HeLa or HTC cells were collected by low speed centrifugation and washed at least two times with cold PBS, pH 7.4, by resuspension and centrifugation. The cells were then washed with 10 mM Hepes, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, and 2 mM DTT and pelleted. The cell pellet was gently resuspended in 1.5 vol of lysis buffer (5 mM Hepes, pH 7.3, 10 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 20  $\mu$ M cytochalasin B, 1 mM PMSF, and 1  $\mu$ g/ml each aprotinin, leupeptin, and pepstatin) and swelled for 10 min on ice. The cells were lysed by five strokes in a tight fitting stainless steel dounce homogenizer. The resulting homogenates were centrifuged at 1,500 g for 15 min to remove nuclei and cell debris. The supernatants were then sequentially centrifuged at 15,000 g for 20 min and 100,000 g for 30 min. The final supernatants were dialyzed extensively with a collodion membrane apparatus (molecular mass cut-off 25,000 D; Schleicher & Schuell, Inc., Keene, NH) against transport buffer (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM

EGTA, 2 mM DTT, and 1  $\mu$ g/ml each aprotinin, leupeptin, and pepstatin) and frozen in aliquots in liquid nitrogen before storage at  $-80^{\circ}\text{C}$ . The protein concentration of HeLa or HTC cell cytosols was 50–60 mg/ml as determined by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Nuclease-treated or untreated reticulocyte lysate was obtained from Promega Biotec (Madison, WI) and processed as described above with the exception that the lysates were centrifuged only at 100,000  $g$  before dialysis and freezing and cytochalasin B was omitted. Reticulocyte lysates used for hexokinase/glucose treatment were not dialyzed and were treated for 15 min at  $30^{\circ}\text{C}$  with 100 U/ml hexokinase and 10 mM glucose before dilution with  $2\times$  transport buffer and transport substrate. The protein concentration of the reticulocyte lysates was  $\sim 50$  mg/ml as determined by Bio-Rad protein assay.

Oocytes were prepared from *Xenopus laevis* by dissection of the ovary into PBS. The ovary was cleaned of extraneous tissue, blotted to remove excess PBS, and placed in a glass dounce homogenizer with 1 vol of 20 mM Hepes, pH 7.3, 100 mM potassium chloride, 2 mM magnesium chloride, 1 mM DTT, and 1  $\mu$ g/ml each aprotinin, leupeptin, and pepstatin. The oocytes were crushed by two strokes of a very loose fitting pestle. The crude lysate was centrifuged at 2,000  $g$  for 10 min and the crude supernatant frozen in aliquots in liquid nitrogen before storage at  $-80^{\circ}\text{C}$ . When needed, aliquots were thawed and further fractionated as described for the other cell extracts.

Yeast cytosols were prepared from a protease-deficient strain of *Saccharomyces cerevisiae* (BJ3505) as follows. An exponentially growing culture was harvested by centrifugation, washed, and lysed in transport buffer by vortexing with glass beads. Alternatively, yeast cells were first converted to spheroplasts with 3 mg/ml Zymolyase (Kirin Breweries, Tokyo, Japan) for 30 min at  $30^{\circ}\text{C}$  in 1 M sorbitol, 25 mM EDTA, 10 mM 2-mercaptoethanol, 40 mM potassium phosphate, pH 7.5. The spheroplasts were collected by centrifugation, washed, and resuspended in 0.3 M mannitol in transport buffer. The spheroplasts were lysed either by five strokes in a tight fitting stainless steel dounce homogenizer or by gentle vortexing in the presence of glass beads. Unbroken cells and cell debris were removed by centrifugation at 1,000  $g$ . Exponentially growing *E. coli* strain JA226 was lysed by sonication in transport buffer and centrifuged as described above. The yeast and bacterial lysates were then centrifuged at high speed and dialyzed against transport buffer as described above. Oocyte and yeast cytosols and bacterial lysates contained  $\sim 50$ –70 mg/ml protein as determined by Bio-Rad protein assay.

### Cell Permeabilization and In Vitro Transport

Cells grown on coverslips were rinsed in cold transport buffer followed by immersion in ice cold transport buffer containing 40  $\mu$ g/ml digitonin (Calbiochem-Behring Corp.; diluted from a 20 mg/ml stock solution in DMSO). The cells were allowed to permeabilize for 5 min after which the digitonin containing buffer was removed and replaced with cold transport buffer. The coverslips were then blotted to remove excess buffer and inverted over a drop of complete transport mixture on a sheet of parafilm in a humidified box. The complete transport mixture contained 50–75% cytosol diluted with transport buffer to give the following final conditions: 25–35 mg/ml protein, 100 nM APC-peptide conjugate, 20 mM Hepes, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM DTT, 1.0 mM EGTA, 1 mM ATP, 5 mM creatine phosphate (Calbiochem-Behring Corp.), 20 U/ml creatine phosphokinase (Calbiochem-Behring Corp.), and 1  $\mu$ g/ml each aprotinin, leupeptin, and pepstatin. The entire box was then floated in a water bath at  $30^{\circ}\text{C}$ . For the WGA inhibition experiment, the coverslip was incubated with transport buffer containing 50  $\mu$ g/ml WGA for 15 min at  $20^{\circ}\text{C}$ . The coverslip was then blotted to remove excess buffer and inverted on a drop of complete transport mix. At the end of the assay, each coverslip was rinsed and mounted on a glass microscope slide in a small amount of transport buffer and the coverslip edges were sealed with nail polish. Samples were observed by phase contrast and epifluorescence with a Zeiss AxioPhot microscope equipped with a  $63\times$  planapochromat objective.

The amount of protein solubilized by digitonin permeabilization of the cells was determined using [ $^{35}\text{S}$ ]methionine-labeled HeLa cells. HeLa cells grown on a 100-mm tissue culture dish were labeled for 14 h with 5  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]methionine in DME containing one half the normal methionine and 5% FBS. Before permeabilization, the cells were again labeled for 3 h with 20  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]methionine in methionine-free medium containing 5% FBS. The cells were rinsed thoroughly in PBS and permeabilized as described above. The extracted material was centrifuged at 12,500  $g$  for 10 min and aliquots of the supernatant were counted in liquid scintillation cocktail. The permeabilized cells were scraped from the plate with a rubber policeman, suspended in a small volume of buffer, and aliquots were counted in liquid scintillation cocktail.

### Time Course of Nuclear Transport

After incubation for the indicated times, coverslips were blotted without rinsing and mounted on a slide in 8  $\mu$ l of the same mix in which the coverslip had been incubated. The slides were then immediately observed in the microscope and photographs were taken with Kodak T-Max film. The fields photographed were chosen essentially at random. Quantitation of the amount of nuclear accumulation of the fluorescent probe (compared to the initial level of nonnuclear fluorescence in the reaction) was determined by densitometric scanning of the negatives with a scanning laser densitometer (LKB Instruments, Inc., Gaithersburg, MD) and comparison to values generated from a standard curve representing different concentrations of the APC-peptide conjugate.

### N-ethylmaleimide (NEM) Treatment

Cytosol fractions to be treated with NEM were first dialyzed in transport buffer containing only 0.5 mM DTT. Treatment of the cytosol was carried out with 5 mM NEM alone or 5 mM NEM + 10 mM DTT for 10 min at  $4^{\circ}\text{C}$  followed by quenching of the NEM-treated sample with 10 mM DTT. The permeabilized cells used to assay the treated fraction were prepared as described above. For NEM treatment of the transporting cells, cells permeabilized as described above were first rinsed with transport buffer without DTT then incubated with transport buffer containing 1 mM NEM or 1 mM NEM + 2 mM DTT for 10 min at  $4^{\circ}\text{C}$ . The coverslips were then rinsed in transport buffer containing 10 mM DTT. Untreated cytosol fraction used with the treated cells was prepared as described above.

### Antibody Staining

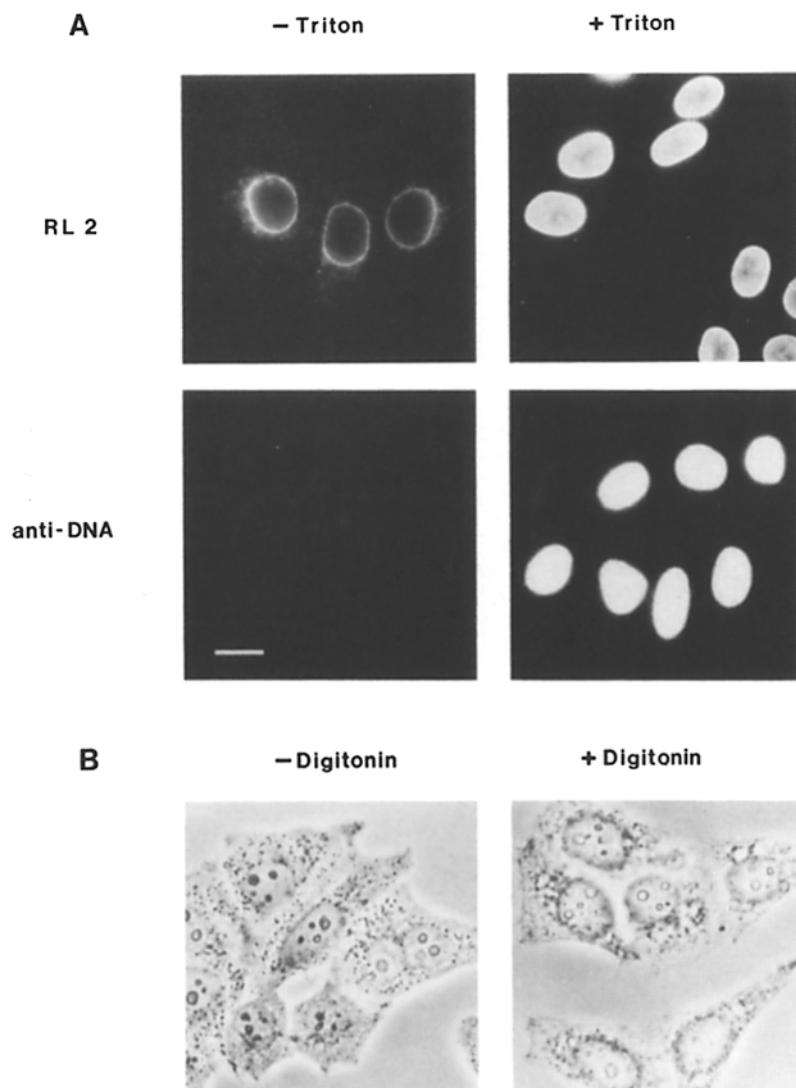
Staining with RL2 (44; a mouse mAb recognizing nuclear pore complex antigens) or with mouse anti-DNA antibodies (27) was carried out by rinsing the coverslips in 0.2% gelatin dissolved in PBS and incubating the coverslips for 15 min at room temperature with the appropriate antibody diluted in gelatin-PBS. The coverslips were rinsed and incubated for an additional 15 min with a secondary antibody conjugated to fluorescein diluted in gelatin-PBS. To examine cells treated with Triton X-100, coverslips were first incubated in PBS containing 0.2% detergent for 6 min at room temperature, and antibody incubations were subsequently performed as described above.

## Results

### Cell Permeabilization

To study the biochemical events in nucleocytoplasmic transport, we have developed a permeabilized cell system that faithfully mimics nuclear protein import seen in the intact cell. Cells are permeabilized with the weak nonionic detergent digitonin, which at low concentrations selectively perforates the plasma membrane releasing cytosolic components from cells while the nuclear envelope and other major membrane organelles remain intact. Digitonin preferentially permeabilizes the plasma membrane compared to internal cellular membranes due to the plasma membrane's proportionally higher cholesterol content (5). Cells permeabilized with digitonin have been used to study various cellular processes, including those involving membranous organelles (3, 30).

The plasma membrane of HeLa cells grown on glass coverslips can be efficiently permeabilized with 40  $\mu$ g/ml of digitonin (Fig. 1). To evaluate the integrity of the plasma membranes and nuclear envelopes of cells after detergent treatment, we incubated unfixed permeabilized cells with either RL2, a monoclonal IgG that reacts with O-linked glycoproteins of the pore complex, or with anti-DNA antibodies. The plasma membranes of detergent-treated cells are freely permeable to macromolecules the size of IgG (Fig. 1), since the antibody RL2 decorates the nuclear envelope (44). However, the nuclear envelope in these cells is structurally intact,



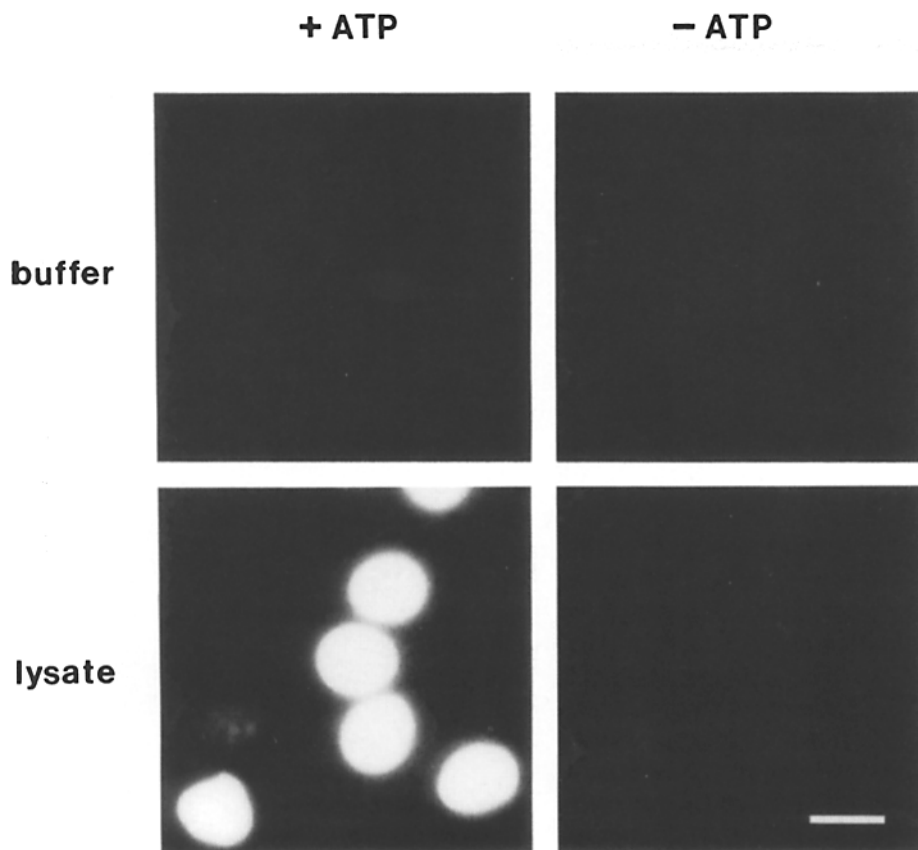
**Figure 1.** The nuclear envelope remains intact after digitonin permeabilization. (A) HeLa cells permeabilized as described in Materials and Methods were stained by indirect immunofluorescence with the antinuclear pore complex antibody RL2 or with anti-DNA antibodies. The permeabilized cells were either washed in buffer alone (*- Triton*) or in 0.1% Triton (*+ Triton*) to remove the nuclear envelope before incubation with the antibodies. (B) Phase-contrast images of HeLa cells without (*-Digitonin*) or with (*+Digitonin*) extraction. Bar, 20  $\mu$ m.

since anti-DNA antibodies, which are too large to diffuse through the pore complex *in vivo*, do not bind to the nuclear interior of the permeabilized cells (Fig. 1). In contrast, when the permeabilized cells are extracted with the nonionic detergent Triton X-100 to disrupt the nuclear envelope before antibody incubation, the nucleus is strongly labeled with the anti-DNA antibody, and RL2 labels intranuclear proteins that it recognizes in HeLa cells in addition to antigens on the cytoplasmic surface of the pore complex. While digitonin treatment releases  $\sim 18\%$  of the total cellular protein (see Materials and Methods), it does not lead to any gross changes in cell morphology detectable by phase-contrast microscopy (Fig. 1). These results demonstrate that using these conditions of digitonin permeabilization, the plasma membrane is perforated to allow release of cytosolic components and access of macromolecules to the nuclear surface, yet the nuclear envelope remains intact.

#### **Cytosol-dependent Nuclear Protein Import in Permeabilized Cells**

We chemically coupled synthetic peptides containing the NLS of the SV40 large T antigen to the naturally fluorescent

protein allophycocyanin (APC) to generate a substrate to study NLS-dependent nuclear protein import. APC has a molecular mass of 104,000, and is too large to enter the nucleus by passive diffusion (4, 11). Therefore, import of this substrate into intact nuclei would require mediated mechanisms. When the APC-peptide conjugate containing the wild type NLS is incubated with permeabilized cells in transport buffer alone or in buffer supplemented with ATP, no nuclear accumulation is observed (Fig. 2). However, if the transport substrate is incubated with permeabilized cells in the presence of cytosol from rabbit reticulocytes, the APC-peptide conjugate is rapidly concentrated in the nucleus (Fig. 2). This accumulation is ATP dependent, since no nuclear import is observed if the reticulocyte lysate is first depleted of ATP by extensive dialysis or by treatment with hexokinase/glucose (Fig. 2). Addition of ATP to the dialyzed cytosol restores transport activity, but addition of GTP alone does not. While the undialyzed reticulocyte cytosol is capable of supporting transport without the addition of exogenous ATP or an ATP regenerating system, cytosol was routinely dialyzed to equilibrate in transport buffer, and exogenous ATP and an ATP regenerating system were added for transport studies.



**Figure 2.** Nuclear transport requires cytosol and ATP in permeabilized HeLa cells. Permeabilized HeLa cells were incubated in transport buffer alone or in complete transport mix containing rabbit reticulocyte cytosol in the presence or absence of ATP. After 30 min at 30°C, only the assay containing cytosol and ATP shows accumulation of the APC-peptide conjugate. Bar, 20  $\mu$ m.

The nuclear envelope remains structurally intact as a diffusion barrier throughout the transport assay. When permeabilized cells are incubated with an anti-DNA antibody after transport, the anti-DNA antibody is completely excluded from the nucleus (Fig. 3). In contrast, the nucleus becomes strongly labeled with the anti-DNA antibody when the nuclear envelope is disrupted with Triton X-100 after the transport reaction (Fig. 3). The APC-peptide conjugate is completely released from nuclei after transport when the nuclear envelope is disrupted with Triton X-100 (Fig. 3) or by mechanical disruption induced by gently shearing coverslips across the mounting slide (data not shown). This demonstrates that retention of the APC-peptide conjugate in the nucleus is due to an intact nuclear envelope rather than binding to intranuclear components. Maintenance of the structural integrity of the nuclear envelope during incubation at 30°C does not depend on the presence of cytosol during the transport reaction, since permeabilized cells incubated with transport buffer alone lacking ATP exclude anti-DNA antibodies added at the end of the transport incubation (data not shown).

The nuclear-associated APC-peptide conjugate in these assays is mainly present in the nuclear interior, as evident from observing the nucleus in successive focal planes. For example, in planes imaging the center of the nucleus, nuclear envelope proteins present a rim distribution in immunofluorescence labeling (Fig. 1), while the APC conjugate is diffusely distributed throughout the nuclear interior (Fig. 2). We do not observe binding of the substrate to nuclear envelopes by fluorescence microscopy when permeabilized cells are incubated with cytosol lacking ATP, in contrast to the results of others (36). This is possibly due to the relatively

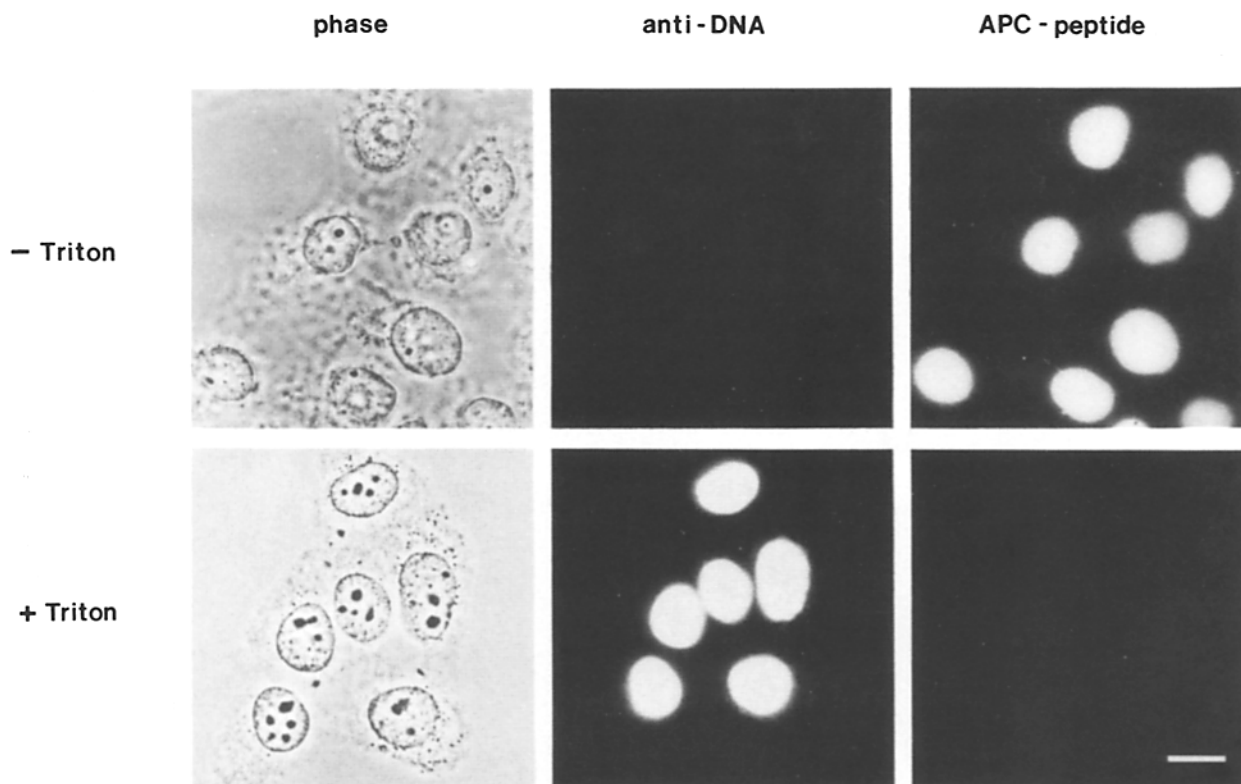
low amount of peptide crosslinked to our transport substrate, compared to the other studies (36; see Discussion).

### **Specificity of Transport**

The transport of the APC-peptide conjugate is specific for a functional NLS, since an APC-peptide conjugate containing a mutant sequence that is severely deficient for nuclear transport does not enter the nucleus, in contrast to the wild type APC conjugate which becomes highly concentrated in the nucleus (Fig. 4). Allophycocyanin modified with only the crosslinking reagent also is unable to enter or accumulate in the nucleus (data not shown). Previous studies have shown that nuclear protein import is inhibited by the lectin WGA both in vivo and in vitro (6, 14, 45, 47), presumably through its interaction with nuclear pore complex proteins containing O-linked *N*-acetylglucosamine residues (7, 21, 22, 23). When permeabilized cells are preincubated in transport buffer containing 50  $\mu$ g/ml WGA before incubation in complete transport mix without added lectin, transport is totally inhibited (Fig. 4). The transport reaction is also temperature dependent. If the assay is carried out at 4°C, no transport is observed. Therefore, ATP hydrolysis is probably necessary for nuclear protein import as previously suggested (36, 41).

### **Time Course of Accumulation**

Nuclear import of proteins synthesized in vivo is very rapid being largely completed within 15 min (42). The nuclei of permeabilized cells also rapidly import a nuclear transport substrate, accumulating up to 30-fold concentration of the APC-peptide conjugate compared to the background fluo-



**Figure 3.** The nuclear envelope remains intact throughout the transport assay. After a 30-min incubation to allow accumulation of APC-peptide, the cells were rinsed either in transport buffer (*-Triton*) or in transport buffer containing 0.2% Triton (*+Triton*) and stained by indirect immunofluorescence with the anti-DNA antibody. Only the nuclei in Triton-extracted cells are accessible to the antibody. Triton extraction also causes the release of accumulated APC-peptide from the nucleus. Bar, 20  $\mu$ m.

rescence within 30 min as determined by quantitative film scanning (Fig. 5). Routinely, greater than 97% of cells on the coverslips are capable of accumulating the APC-peptide conjugate to high levels after a 30-min incubation, and at least 35% of the nuclei have accumulated the substrate to levels greater than 20-fold over background. Of the remaining cells that do not transport to significant levels, approximately half accumulate small amounts of substrate while the remainder do not accumulate the substrate. Most nuclei of permeabilized cells import substrate at a roughly similar rate, as shown in the histograms in Fig. 5 where the amount of APC-peptide conjugate accumulated in a population of nuclei after different incubation periods is shown. There is no apparent lag phase in transport, since by 5 min after addition of the transport mix to the cells, accumulation to an average of fourfold above background is observed (Fig. 5). Even after only 1 min, significant transport has occurred so that the nuclei have accumulated the conjugate to concentrations slightly higher than the background fluorescence (data not shown).

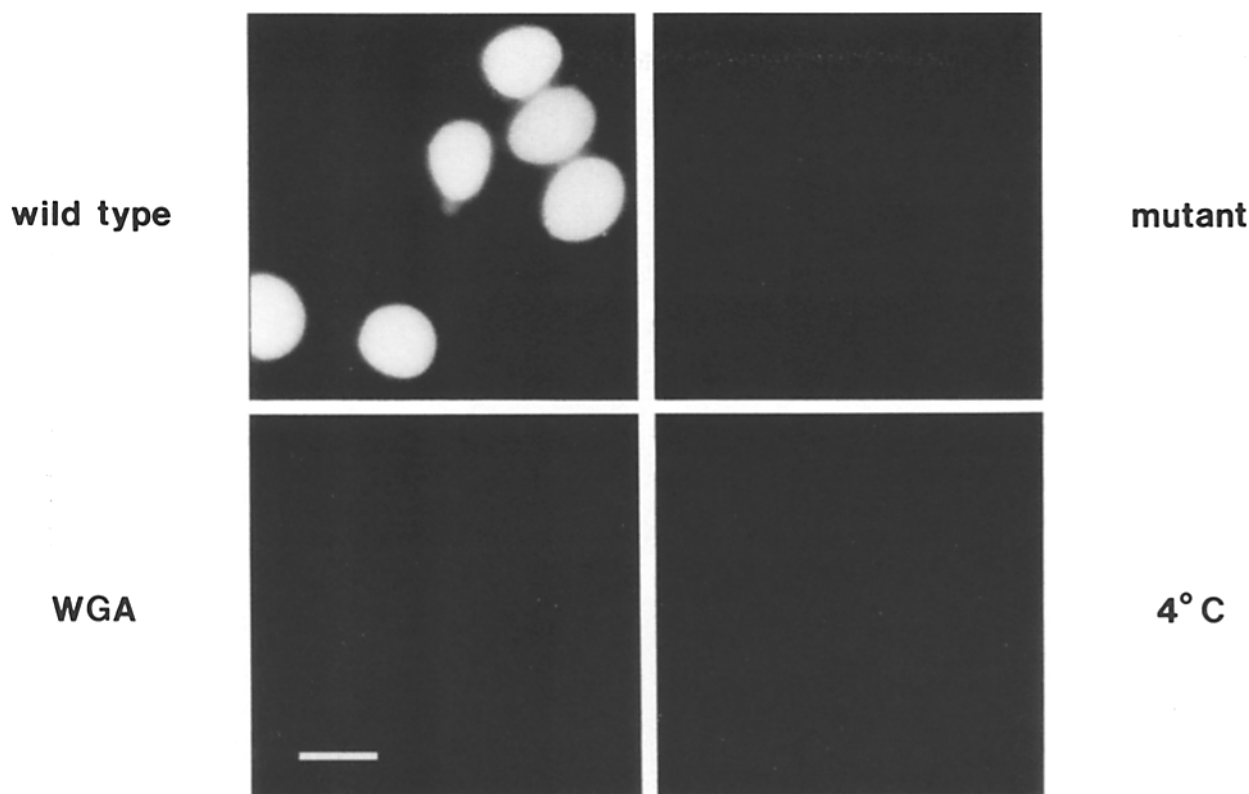
#### **Application to Different Cell Types**

An important attribute to the nuclear import system we have described is its ability to be adapted to a number of different vertebrate cell types and species. As shown in Table I, a number of different combinations of permeabilized cells and cytosol fractions successfully support nuclear protein import. While rabbit reticulocyte lysate is routinely used as the source of cytosol, this can be substituted with comparable

results by cytosols prepared from HeLa cells, HTC cells (a rat hepatoma cell line), or *Xenopus* oocytes. Also, both HeLa and NRK cells (a rat liver cell line) can provide a source of permeabilized cells that are active in nuclear protein import. Therefore, the required cytosolic factors appear to be exchangeable among the vertebrate cells we have tested. With our conditions, cytosol from the yeast *S. cerevisiae* do not support nuclear protein import in permeabilized mammalian cells. It is unlikely that this is due to the inability of yeast factors to recognize the SV40 large T antigen NLS, since this sequence has been shown to be functional in yeast both in vivo (35) and in vitro (43).

#### **Characterization of Transport Factors**

Preliminary characterization of the soluble transport factors present in dialyzed cytosol reveals that they are not pelletable at 100,000 *g* (see Materials and Methods). The factor cannot be nondialyzable ATP, as ATP alone does not allow transport, even in the presence of concentrated protein solutions (Fig. 2 and Table I). It is unlikely that calcium is required for transport in this assay since the assay is normally carried out in the presence of 1 mM EGTA and lysates prepared in the presence of 10 mM EGTA are capable of transport (data not shown). Preliminary results indicate that the cytosolic factor does not contain any RNA, or at least one that is sensitive to digestion with micrococcal nuclease. Reticulocyte lysates treated by the manufacturer to remove mRNA are fully functional for transport as are crude lysates.



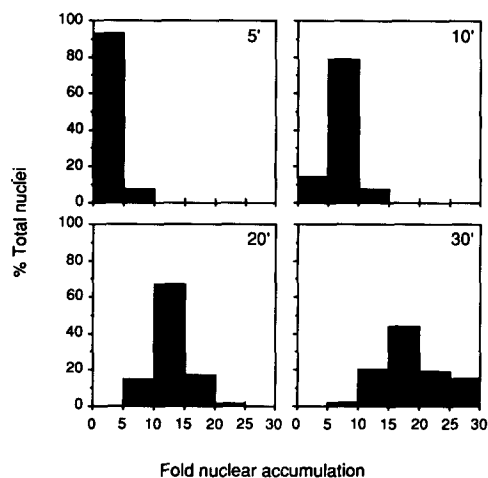
**Figure 4.** Specificity of nuclear transport. Transport assays were carried out as in Fig. 2. An APC-peptide conjugate containing a mutant NLS peptide is defective for transport. Preincubation of the permeabilized cells with WGA completely inhibits transport as does incubation of the complete assay at 4°C. Bar, 20  $\mu$ m.

Pretreatment of either the cytosol or permeabilized cells with the sulfhydryl alkylating reagent NEM inactivates subsequent nuclear protein import (Fig. 6). As expected, addition of DTT along with the NEM completely blocks the

effects of the inhibitor. While treatment of permeabilized cells with as low as 1 mM NEM completely abolishes the capacity for nuclear import, 5 mM NEM is required to inactivate the cytosol. Therefore, it is most likely that at least two NEM-sensitive factors are involved in nuclear import having different sensitivities to NEM inactivation, one in the soluble cytosol and a second in the permeabilized cells (Fig. 6). Since the untreated cytosol is unable to rescue transport in NEM-treated permeabilized cells, and untreated cells do not support transport with NEM-treated cytosol, it is unlikely that the NEM-sensitive factors present in the cytosol and the permeabilized cells are identical.

## Discussion

In this paper we describe a novel cell-free system to study nuclear protein import using cultured mammalian cells that

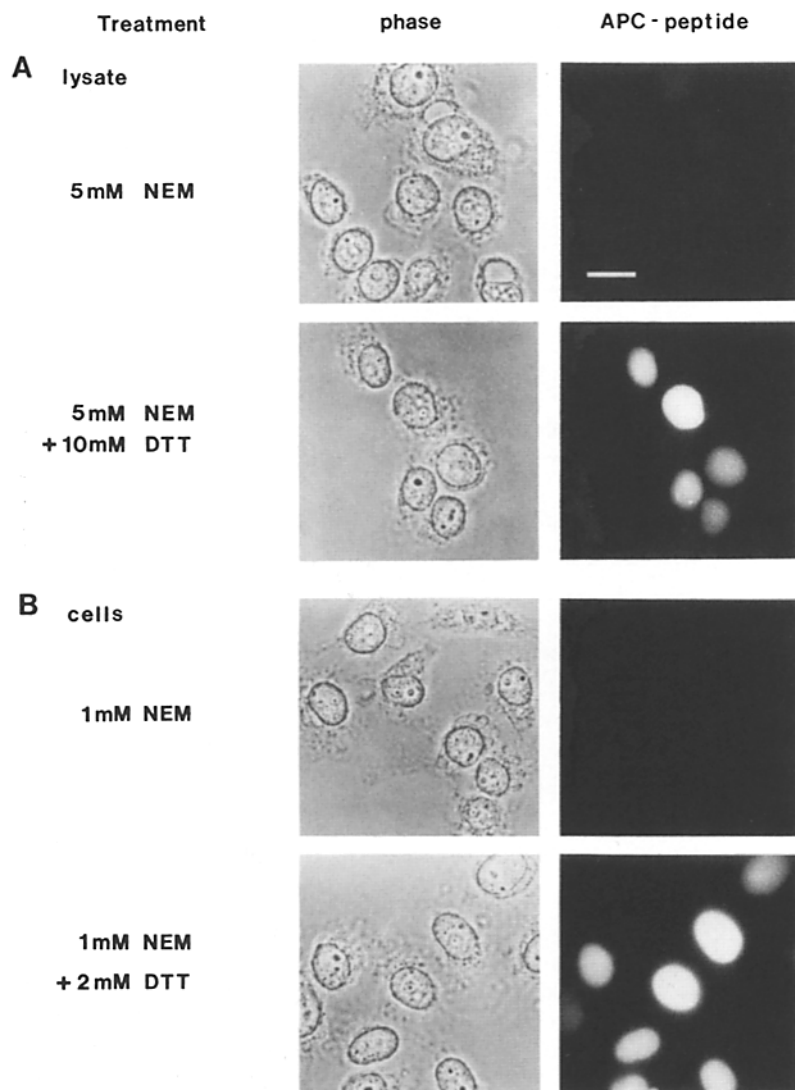


**Figure 5.** Time course of nuclear accumulation of APC-peptide conjugate. Quantitation of nuclear accumulation of the APC-peptide conjugate at various times is described in Materials and Methods. For each time point, the number of nuclei scanned and counted is 5' = 74, 10' = 63, 20' = 75, 30' = 100. Fold concentration is determined by comparing the density value of each nucleus to a standard curve prepared from photographic negatives of different known concentrations of the transport substrate. It should be noted that at the 5' time point, 87% of the cells have accumulated substrate to greater than threefold over background.

**Table I. Heterologous Cell Lysates Support Nuclear Import**

S100 lysates	Permeable cells	
	HeLa	NRK
Rabbit reticulocyte	+	+
HeLa	+	+
HTC	+	+
<i>X. laevis</i> oocyte	ND	+
<i>S. cerevisiae</i>	—	—
<i>E. coli</i>	—	—

+, Efficient transport; —, no transport.



**Figure 6.** Identification of NEM-sensitive activities. (A) Reticulocyte cytosol was treated with NEM followed by quenching with DTT as described in Materials and Methods. As a control, DTT was added to the extract before the NEM (NEM + DTT). (B) The permeabilized cells were treated with NEM followed by quenching with DTT before the addition of untreated assay mix. The control experiment was performed as in A. The cytosol and the cells have different sensitivities to NEM treatment. Bar, 20  $\mu$ m.

have been permeabilized with digitonin. While digitonin treatment efficiently renders the plasma membranes of mammalian cells permeable to macromolecules, the integrity of the nuclear envelope and many other cellular structures are preserved with our extraction conditions. Transport is dependent on supplementing the permeabilized cells with cytosol, indicating that soluble cytoplasmic factors released during cell permeabilization are required for transport of proteins from the cytoplasm to the nucleus. Accumulation of proteins in the nucleus in this system is specific for proteins containing a functional NLS such as the wild type SV40 large T antigen NLS, but not for a protein containing a non-functional mutant NLS. Furthermore, the accumulation is ATP and temperature dependent and can be inhibited by the lectin WGA which binds to pore complexes and inhibits nuclear import in vivo. Thus, the features of this permeabilized cell system closely resemble the characteristics of nuclear protein import seen in intact cells. They also are similar to many features of nuclear protein import obtained with a cell free system that utilizes *Xenopus* egg extracts to assemble or reassemble nuclei.

While several biochemical components of the nuclear pore

complex have been identified recently (7, 19, 44), little is known about the mechanism for translocation of proteins across the nuclear envelope and the involvement of specific pore complex proteins and nonnuclear components in this process (10). Initial attempts to dissect the process of nuclear import into distinct steps have identified potential intermediates in this process. In the absence of ATP and at reduced temperature, NLS-containing substrates with a high density of NLS associate with the pore complex but are not imported into the nucleus both in vivo (41) and in vitro (36). Similarly, pore complex-bound transport ligand was observed when transport was blocked by preincubation with WGA (14). Whether these pore complex-bound ligands are true biochemical intermediates that can be imported into the nucleus upon subsequent incubation under nonrestrictive conditions has not been experimentally determined. In the work described here, no significant association of transport substrate with the nuclear envelope is observed by fluorescence microscopy in the absence of ATP or when transport is inhibited by WGA. The transport substrates used in this study have a low peptide to protein ratio (four to eight peptides/protein) which probably more accurately reflects the

state of native proteins in the cell. We have not determined whether pore complex binding can be detected using ligands with increased signal sequence to protein ratios, which is reported to enhance binding (36).

A recent reported described two NEM-sensitive cytosolic factors necessary for obtaining nuclear protein import in a cell-free system involving incubation of isolated rat liver nuclei with *Xenopus* egg extracts (37). It has not been determined whether the cytosolic factors in this system are required to make the isolated rat nuclei competent to import proteins, or whether they are involved in the transport process itself. In addition to resealing the isolated rat nuclei, the *Xenopus* extract also may supply nuclear factors lost from the rat nuclei during preparation, such as nuclear pore complex components that are abundantly present in the *Xenopus* nuclear assembly extract (13, 33, 39).

The cytosolic factors described in this study are unlikely to be involved in nuclear envelope assembly or stability since the nuclear envelope in the digitonin permeabilized cells remains intact throughout the transport assay, even when cytosol is omitted. Furthermore, only very small amounts of unassembled nuclear envelope proteins are present in the interphase cell cytosol used to support transport in permeabilized cells. Thus, it is very unlikely that the added interphase cytosol modifies preexisting nuclear envelope structure in the permeabilized cells, in contrast to *Xenopus* egg extracts. The transport factors are removed from the cells by gentle permeabilization of the plasma membrane and are therefore likely to be freely soluble in the cytoplasm or very loosely associated with intracellular structures. The absence of a lag phase and the rapid nuclear accumulation of substrate in the permeabilized cell system suggests that the cytosolic factors are not involved in forming supramolecular complexes, or that they do so very quickly. Since the factors can be supplied back to the cells by an extract made from anucleate cells (rabbit reticulocytes), they are likely to be truly cytoplasmic. However, it cannot be totally excluded that they are released from the nucleus during maturation of the reticulocyte.

The results presented here differ from two earlier reports (24, 34) which suggested that isolated rat liver nuclei in buffer alone are capable of nuclear protein import. While these reports described an NLS-dependent association of proteins with nuclei, it is not clear whether these systems measured transport of substrate across the nuclear envelope of intact nuclei, or binding of substrate to the nuclear pore complex and/or nuclear contents of nuclei that are leaky. Since NLS binding proteins apparently are present in the nuclear contents as well as cytoplasm (1, 32, 46), signal-dependent association of proteins with nuclei cannot be interpreted to suggest transport through the pore complex unless the nucleus is shown to be structurally intact. In the latter case, entry into the nucleus would be restricted to mediated transport through the pore complex. If authentic nuclear protein import occurred in these studies, cytoplasmic factors may have contributed to nuclear accumulation of substrate due to significant cytoplasmic contamination of the nuclear preparations. Recently, *in vitro* association of NLS-containing substrates with isolated yeast nuclei was reported that was dependent on ATP and calcium (26). Small amounts of reticulocyte lysate were present in the assays, and could have supplied necessary factors for the observed nuclear association.

Using synthetic peptides as probes, a variety of proteins that bind nuclear location sequence have been identified in mammalian and yeast cells (1, 31, 32, 46). Two polypeptides of molecular mass 60 and 70 kD of rat liver that bind the SV40 large T antigen nuclear location sequence with high affinity and specificity were found to be in both the cytoplasm and nucleus, suggesting that they may function as receptors for protein import that shuttle between the cytoplasm and the nucleus (1). A cell physiological approach has also suggested the existence of cytoplasmic binding sites for NLS (4). The relationship of these NLS binding proteins and the soluble factors identified here is currently being investigated.

It has been proposed that 3–4 nm diameter fibers seen by electron microscopy emanating from the cytoplasmic face of the nuclear pore complex in oocytes may be involved in transport (15–17). If these fibers exist in the somatic cells of higher eukaryotes, the permeabilized cells described here should preserve the structure of these components and their spatial relationship to other cellular components. Thus, it should be possible in future studies to analyze the functions of these fibers and their possible relationship to other transport factors in this permeabilized cell system.

In conclusion, the permeabilized cell system we have described to analyze nuclear protein import should prove particularly useful in future work to identify soluble factors involved in transport, to characterize intermediate steps in the process, and to define other biochemical features of this process. This system also may prove useful for analyzing RNA export from the nucleus. Since the system can utilize homologous components and is not dependent on repair of isolated nuclei, biochemical dissection of components required specifically for nuclear transport should be facilitated.

The authors would like to acknowledge Dr. James Glass for comments on the manuscript. We would also like to thank Drs. Robert Rubin and Eng Tan for their generous gift of anti-DNA antibodies.

This work was supported by a grant from the National Institutes of Health (to L. Gerace) and a fellowship from the Damon Runyon-Walter Winchell Cancer Research Fund (to S. A. Adam). We are particularly grateful for support from the G. Harold and Leila Y. Mathers Charitable Foundation.

Received for publication 10 May 1990 and in revised form 24 May 1990.

## References

1. Adam, S. A., T. J. Lobl, M. A. Mitchell, and L. Gerace. 1989. Identification of specific binding proteins for a nuclear location sequence. *Nature (Lond.)* 337:276–279.
2. Akey, C. W., and D. S. Goldfarb. 1989. Protein import through the nuclear pore complex is a multistep process. *J. Cell Biol.* 109:971–982.
3. Bittner, M. A., and R. W. Holz. 1988. Effects of tetanus toxin on catecholamine release from intact and digitonin-permeabilized chromaffin cells. *J. Neurochem.* 51:451–456.
4. Breeuwer, M., and D. S. Goldfarb. 1990. Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. *Cell* 60:999–1008.
5. Colbeau, A., J. Nachbaur, and P. M. Vignais. 1971. Enzymic characterization and lipid composition of rat liver subcellular membranes. *Biochim. Biophys. Acta* 249:462–492.
6. Dabauvalle, M.-C., B. Schulz, U. Scheer, and R. Peters. 1988. Inhibition of nuclear accumulation of karyophilic proteins in living cells by microinjection of the lectin wheat germ agglutinin. *Exp. Cell Res.* 174:291–296.
7. Davis, L. I., and G. Blobel. 1987. Nuclear pore complex contains a family of glycoproteins that includes p62: glycosylation through a previously unidentified cellular pathway. *Proc. Natl. Acad. Sci. USA* 84:7552–7556.
8. Dworetzky, S. I., and C. M. Feldherr. 1988. Translocation of RNA-coated gold particles through the nuclear pores of oocytes. *J. Cell Biol.* 106:575–584.
9. Dworetzky, S. I., R. E. Lanford, and C. M. Feldherr. 1988. The effect of

- variations in the number and sequence of targeting signals on nuclear uptake. *J. Cell Biol.* 107:1279-1288.
10. Featherstone, C., M. K. Darby, and L. Gerace. 1988. A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA in vivo. *J. Cell Biol.* 107:1289-1297.
  11. Feldherr, C. M., R. J. Cohen, and J. A. Ogburn. 1983. Evidence for mediated protein uptake by amphibian oocyte nuclei. *J. Cell Biol.* 96:1486-1490.
  12. Feldherr, C. M., E. Kallenbach, and N. Schultz. 1984. Movement of a karyophilic protein through the nuclear pore of oocytes. *J. Cell Biol.* 99:2216-2222.
  13. Finlay, D. R., and D. J. Forbes. 1990. Reconstitution of biochemically altered nuclear pores: transport can be eliminated and restored. *Cell.* 60:17-29.
  14. Finlay, D. R., D. D. Newmeyer, T. M. Price, and D. J. Forbes. 1987. Inhibition of in vitro nuclear transport by a lectin that binds to nuclear pores. *J. Cell Biol.* 104:189-200.
  15. Franke, W. W. 1970. On the universality of the nuclear pore complex structure. *Z. Zellforsch. Mikrosk. Anat.* 105:405-429.
  16. Franke, W. W., and U. Scheer. 1970. The ultrastructure of the nuclear envelope of amphibian oocytes: a reinvestigation. I. The mature oocyte. *J. Ultrastruct. Res.* 30:288-316.
  17. Franke, W. W., U. Scheer, G. Krohne, and E. D. Jarasch. 1981. The nuclear envelope and the architecture of the nuclear periphery. *J. Cell Biol.* 91:39s-50s.
  18. Gerace, L., and B. Burke. 1988. Functional organization of the nuclear envelope. *Annu. Rev. Cell Biol.* 4:335-374.
  19. Gerace, L., Y. Ottaviano, and C. Kondor-Koch. 1982. Identification of a major polypeptide of the nuclear pore complex. *J. Cell Biol.* 95:826-837.
  20. Goldfarb, D. S., J. Gariepy, G. Schoolnik, and R. D. Kornberg. 1986. Synthetic peptides as nuclear localization signals. *Nature (Lond.)* 326:641-644.
  21. Hanover, J. A., C. K. Cohen, M. C. Willingham, and M. K. Park. 1987. O-linked N-acetyl glucosamine is attached to proteins of the nuclear pore: evidence for cytoplasmic and nucleoplasmic glycoproteins. *J. Biol. Chem.* 262:9887-9894.
  22. Holt, G. D., and G. W. Hart. 1986. The subcellular distribution of terminal N-acetylglucosamine moieties: localization of a novel protein-saccharide linkage, O-linked GlcNAc. *J. Biol. Chem.* 261:8049-8057.
  23. Holt, G. D., C. M. Snow, A. Senior, R. S. Haltiwanger, L. Gerace, and G. W. Hart. 1987. Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked N-acetylglucosamine. *J. Cell Biol.* 107:1157-1164.
  24. Imamoto-Sonobe, N., Y. Yoneda, R. Iwamoto, H. Sugawa, and T. Uchida. 1988. ATP-dependent association of nuclear proteins with isolated rat liver nuclei. *Proc. Natl. Acad. Sci. USA.* 85:3426-3430.
  25. Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear location. *Cell.* 39:499-509.
  26. Kalinich, J. F., and M. G. Douglas. 1989. In vitro translocation through the yeast nuclear envelope. *J. Biol. Chem.* 264:17979-17989.
  27. Kotzin, B. L., J. A. Lafferty, J. P. Portanova, R. L. Rubin, and E. M. Tan. 1984. Monoclonal anti-histone autoantibodies derived from murine models of lupus. *J. Immunol.* 133:2554-2559.
  28. Lanford, R. E., and J. S. Butel. 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. *Cell.* 37:801-813.
  29. Lanford, R. E., P. Kanda, and R. C. Kennedy. 1986. Induction of nuclear transport with a synthetic peptide homologous to the SV40 T antigen transport signal. *Mol. Cell. Biol.* 8:2722-2729.
  30. Lazarovici, P., K. Fujita, M. L. Contreras, J. P. DiOrto, and P. I. Lelkes. 1989. Affinity purified tetanus toxin binds to isolated chromaffin granules and inhibits catecholamine release in digitonin-permeabilized chromaffin cells. *FEBS (Fed. Eur. Biochem. Sci.) Lett.* 253:121-128.
  31. Lee, W.-C., and T. Melese. 1989. Identification and characterization of a nuclear localization sequence-binding protein in yeast. *Proc. Natl. Acad. Sci. USA.* 86:8808-8812.
  32. Li, R., and J. O. Thomas. 1989. Identification of a human protein that interacts with nuclear localization signals. *J. Cell Biol.* 109:2623-2632.
  33. Lohka, M. J., and Y. Masui. 1984. Roles of and cytoplasmic particles in nuclear envelope assemble and sperm pronuclear formation in cell-free preparations from amphibian eggs. *J. Cell Biol.* 98:1222-1230.
  34. Markland, W., A. E. Smith, and B. L. Roberts. 1987. Signal-dependent translocation of Simian Virus 40 large T antigen into rat liver nuclei in a cell-free system. *Mol. Cell. Biol.* 7:4255-4265.
  35. Nelson, M., and P. Silver. 1989. Context affects nuclear protein localization in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 9:384-389.
  36. Newmeyer, D. D., and D. J. Forbes. 1988. Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. *Cell.* 52:641-653.
  37. Newmeyer, D. D., and D. J. Forbes. 1990. An N-ethylmaleimide-sensitive cytosolic factor necessary for nuclear protein import: requirement in signal-mediated binding to the nuclear pore. *J. Cell Biol.* 110:547-557.
  38. Newmeyer, D. D., D. R. Finlay, and D. J. Forbes. 1986. In vitro transport of a fluorescent nuclear protein and exclusion of non-nuclear proteins. *J. Cell Biol.* 103:2091-2102.
  39. Newmeyer, D. D., J. M. Lucocq, T. R. Burglin, and E. M. DeRobertis. 1986. Assembly in vitro of nuclei active in nuclear protein transport: ATP is required for nucleoplasmic accumulation. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:501-510.
  40. Paine, P. L., L. C. Moore, and S. B. Horowitz. 1975. Nuclear envelope permeability. *Nature (Lond.)* 254:109-114.
  41. Richardson, W. D., A. D. Mills, S. M. Dilworth, R. A. Laskey, and C. Dingwall. 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through the nuclear pores. *Cell.* 52:655-664.
  42. Schickendantz, J., K. H. Scheidtmann, and G. Walter. 1986. Kinetics of nuclear transport and oligomerization of simian virus 40 large T antigen. *Virology.* 148:47-57.
  43. Silver, P., I. Sadler, and M. A. Osborne. 1989. Yeast proteins that recognize nuclear localization sequences. *J. Cell Biol.* 109:983-989.
  44. Snow, C. M., A. Senior, and L. Gerace. 1987. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. *J. Cell Biol.* 104:1143-1156.
  45. Wolff, B., M. C. Willingham, and J. A. Hanover. 1988. Nuclear protein import: specificity for transport across the nuclear pore. *Exp. Cell Res.* 178:318-334.
  46. Yamasaki, L., P. Kanda, and R. E. Lanford. 1989. Identification of four nuclear transport signal-binding proteins that interact with diverse transport signals. *Mol. Cell. Biol.* 9:3028-3036.
  47. Yoneda, Y., N. Imamoto-Sonobe, M. Yamaizumi, and T. Uchida. 1987. Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. *Exp. Cell Res.* 173:586-595.