Journal of Cell Science 108, 1849-1861 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

The mechanism of nuclear transport of natural or artificial transport substrates in digitonin-permeabilized cells

Imre Cserpán and Andor Udvardy*

Biological Research Center of the Hungarian Academy of Sciences, 6701 Szeged, PO Box 521, Hungary

*Author for correspondence

SUMMARY

Characterization of nuclear protein transport in digitonin-permeabilized cells revealed that the number of the nuclear localization signal sequences (NLS) within the transport substrate basically influences the mechanism of the transport reaction.

Phycoerythrine-NLS transport substrate carrying a maximum of 4-5 conjugated NLSs/subunit, or Bsp methyltransferase-NLS fusion protein were efficiently transported into the nuclei of digitonin-permeabilized cultured cells without any exogenously added cytosolic protein. All the characteristic properties of in vivo nuclear transport are faithfully reproduced with these transport substrates: (i) the transport requires a functional NLS in the transported protein, a transport-incompetent mutant NLS being ineffective; (ii) the transport is energy dependent; (iii) the wild type nuclear localization peptide efficiently competes for transport, while the transport-incompetent mutant peptide does not; and (iv) wheat germ agglutinin inhibits this transport reaction. Nuclear transport observed with these substrates was not due to any damage of the nuclear membrane or inefficient extraction of the cytosolic proteins during the permeabilization of the cells. The nuclear transport was proportional to the number of conjugated NLSs.

Nuclear transport of phycoerythrine carrying 7-8 conjugated NLSs/subunit required the addition of exogenous cytosolic proteins. This transport also fulfilled all the characteristic properties of an authentic nuclear transport.

Nuclear transport with different combinations of transport substrates further supported the assumption that distinct transport mechanisms operate for different substrates. From a mixture of PE-NLS$_{7-8}$ and Bsp methyltransferase-NLS, the highly conjugated substrate was completely retained in the cytoplasm in the absence of exogenous cytosol, while Bsp methyltransferase-NLS was efficiently transported. Exogenous cytosol promoted the nuclear transport of the highly conjugated substrate.

Key words: nuclear transport, nuclear localization signal, digitonin permeabilization

INTRODUCTION

The mechanism of protein transport to the nucleus has been studied by a large variety of in vivo and in vitro techniques (for reviews, see Dingwall, 1991; Garcia-Bustos et al., 1991; Siver, 1991; Nigg et al., 1991; Hanover, 1992; Dingwall and Laskey, 1992; Osborn and Silver, 1993; Newmeyer, 1993; Hurt, 1993). Microinjection of living cells (one of the most important in vivo techniques) revealed that small molecular mass dextrans or various small proteins lacking an NLS diffuse freely across the nuclear pore complex (Bonner, 1975; Paine et al., 1975; Peters, 1986). Proteins larger than 30-40 kDa, however, cannot diffuse rapidly across the channel of the nuclear pore complex, and appear to be transported from the cytoplasm into the nucleus by a selective, mediated mechanism (Feldherr et al., 1983). Microinjection studies demonstrated that this active transport depends on the presence of a short sequence in the protein, which is called the nuclear localization signal, and involves two steps: the rapid binding of proteins having the NLS sequence at the nuclear envelope, and a slower translocation step through the nuclear pores (Richardson et al., 1988). The first step in this process is energy-independent, while the translocation through the nuclear pore requires ATP and can be completely blocked by cooling or energy depletion (Newmeyer et al., 1986a,b; Richardson et al., 1988; Breeuwer and Goldfarb, 1990). Microinjection of living cells also revealed that histone H1 and other nucleophilic proteins small enough to pass through the nuclear pore by passive diffusion, are also transported by the active, ATP-dependent facilitated mechanism (Breeuwer and Goldfarb, 1990).

The transport of NLS-containing large proteins into the nucleus displays saturable kinetics (Goldfarb et al., 1986), suggesting that the transport is a receptor-mediated process. To identify, isolate and characterize this presumed NLS receptor(s), and to dissect the nuclear transport process into greater detail, in vitro nuclear protein transport systems have been established. The first such system utilized an extract of unfertilized *Xenopus* egg, which contains all the components required for the assembly of an intact nucleus around naked DNA or chromatin (Newport, 1987; Newmeyer et al., 1986a,b). The two steps of nuclear protein transport, binding

of the NLS-containing protein at the nuclear membrane in the absence of ATP, and translocation through the nuclear pore in the presence of ATP, were faithfully reproduced in this in vitro system (Newmeyer and Forbes, 1988). Furthermore, a cytoplasmic component (nuclear import factor I) was detected, which is involved in the NLS-mediated binding to the nuclear pore (Newmeyer and Forbes, 1990).

Digitonin-permeabilized cultured cells were recently introduced for study of the transport and nuclear accumulation of proteins containing the SV40 large T antigen nuclear localization sequence (Adam et al., 1990). Digitonin permeabilizes the plasma membrane for macromolecules, leaving the nuclear membrane intact. During permeabilization, cytoplasmic proteins involved in the nuclear transport process were reported to be released from the cell, and thus the transport of any synthetic transport substrate, usually a fluorescent protein conjugated with the SV40 large T antigen NLS, required the addition of exogenous cytosol. Nuclear transport in the permeabilized cells required a functional NLS. A single amino acid change in the NLS at lysine 128, which is known to inactivate the in vivo function of the SV40 large T antigen NLS, abolished the nuclear accumulation of the synthetic transport substrate. Furthermore, the transport process in the permeabilized cells required ATP, and it was blocked by wheat germ agglutinin (WGA), indicating that the permeabilized cell system authentically reproduces the nuclear protein import. Due to the simplicity and efficiency of this test system, it was used to test the function of purified NLS-binding proteins in the transport process (Adam and Gerace, 1991; Stochaj and Silver, 1992), to study the interaction of O-linked glycoproteins of the nuclear pore complex with a cytosolic factor required for nuclear protein import (Sterne-Marr et al., 1992), to detect and purify cytosolic proteins required in different steps of the nuclear protein transport (Shannon Moore and Blobel, 1992, 1993; Melchior et al., 1993), to analyze the effect of the 70 kDa heat shock protein in the nuclear transport (Shi and Thomas, 1992), and to study the relevance of the phosphorylation of protein kinase C sites of lamin B_2 in the nuclear transport (Hennekes et al., 1993).

In the present study, nuclear transport in digitonin-permeabilized cells was analyzed by using highly purified synthetic substrates, prepared by two different heterobifunctional cross-linkers, or by cloning an oligonucleotide coding for the SV 40 large T NLS into a bacterial protein. It is shown that the nuclear transport with these substrates show distinct differences, providing further insights into the details of the nuclear transport mechanism.

MATERIALS AND METHODS

Synthesis and purification of peptides

The wild type SV40 large T antigen nuclear localization sequence (Cys-Gly-Gly-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp) and a transport-incompetent mutant sequence (Cys-Gly-Gly-Gly-Pro-Lys-Thr-Lys-Arg-Lys-Val-Glu-Asp), which will be called NLS-Thr 128, containing threonine instead of lysine at position 128 in the T antigen (Kalderon et al., 1984; Goldfarb et al., 1986; Lanford and Butel, 1984), were synthesized on an Advanced ChemTech Model 200 peptide synthesizer according to the manufacturer's recommendations, using well-established solid-phase methods (Stewart and Young, 1984). The protected peptides were cleaved by using anhydrous liquid hydrogen fluoride, exchanged to the acetate form on AG-3 (AcO^- form) anion exchange resin, and purified by reverse phase high performance liquid chromatography on a Delta Pak C18-300 A (Waters) column.

Synthesis and purification of artificial transport substrates

Phycoerythrine (PE) was a kind gift from M. Debreczeny and B. Szalontay (Institute of Biophysics, Biological Research Center, Szeged, Hungary). The protein has a subunit structure of $(ab)_3$ with a native molecular mass of 132 kDa (subunit a=21 kDa, subunit b=23 kDa). The protein was stored as an ammonium sulfate precipitate at 4°C in the dark. The precipitated protein was pelleted at 4°C in a microcentrifuge (5 minutes at 12,000 rpm) and dissolved in 50 mM potassium phosphate, pH 7.8, containing 1 mM EDTA. The ammonium sulfate was removed on a 0.6×10 cm Sephadex G 25 fine column equilibrated with 50 mM potassium phosphate, pH 7.8, 1 mM EDTA, and the concentration was adjusted to 2 mg/ml (the concentration was calculated from the optical density (A) at 565 nm: concentration (mg/ml)= $0.0787 * A_{565}$).

The heterobifunctional cross-linker MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; Pierce) and SMCC (succinimidyl 4-(*N*-maleimidomethyl) cyclohexane 1-carboxylate; Pierce) were dissolved in dimethylsulfoxide at a concentration of 20 mg/ml. 200 μl of purified PE was incubated at 22°C for 1 hour with 10 μl of the appropriate cross-linker, and the activated proteins were purified on a 0.6 × 12 cm Sephadex G 25 fine column equilibrated with 50 mM potassium phosphate, pH 6.7, containing 1 mM EDTA.

The synthetic peptides were incubated at room temperature for 10 minutes in 20 mM Hepes buffer, pH 7.3, 50 mM dithiothreitol, and purified on a 0.5×7 cm Sephadex G 10 column equilibrated with 50 mM potassium phosphate, pH 6.7, containing 1 mM EDTA. The activated PE and the reduced peptide were mixed and incubated at room temperature for 3 hours. At the end of the cross-linking reaction, unreacted cross-linkers were blocked by incubating the reaction mixture in the presence of 10 mM beta-mercaptoethanol and 20 mM Tris-Cl, pH 7.6, for 20 minutes, and the PE-NLS conjugate was purified on a 0.9×15 cm Sephadex G 25 fine column equilibrated with 0.1 M NaCl containing 20 mM Hepes buffer, pH 7.3, and 1 mM EDTA. At this stage of the purification, the PE-NLS conjugate still contained a substantial amount of small molecular mass impurities derived from the hydrolysis of the cross-linker. These impurities appeared on an SDS-polyacrylamide gel after silver staining as a diffuse band in the front, or they could be detected by reverse phase HPLC on a Delta Pack C18 300 column (Waters). These small molecular mass contaminants severely inhibit the nuclear transport in digitonin permeabilized cells in the absence of exogenously added cytosolic proteins. The impurities can be efficiently removed by three cycles of concentration-dilution in a Centricon-10 (Amicon) microconcentrator. After each concentration cycle, the protein conjugate was diluted in 0.6 ml of 0.1 M NaCl, 20 mM Hepes, pH 7.3, and 1 mM EDTA. After the last concentration step, the conjugated protein was diluted to 0.25 mg/ml and stored in this buffer at 4°C in the dark. There are two possibilities to achieve different levels of NLS conjugation: either the peptide is added in increasing molar excess in the second phase of the reaction, or in the first phase of the reaction, the substrate protein is activated by the cross-linker at different pH. Increasing the pH of the buffer between 6.7 and 8.0 greatly enhances the activation level of the substrate protein. The average number of conjugated NLSs can be estimated by SDS-polyacrylamide gel analysis (Fig. 1A). During the coupling reaction, the subunits of PE are also partially cross-linked, producing a ladder of NLS-PE conjugates in the 40 and 60 kDa molecular mass range on a SDS-polyacrylamide gel (Fig. 1A).

Oligonucleotide synthesis, cloning and purification of a natural transport substrate

The 54-mer oligonucleotide 5′-TCA GGG CCC AAG AAG AAA AGG AAG GTG GAG GAC CCT TAC CTT ACC GGT ACC CCG-

3′, and its complementary sequence, were synthesized on a Pharmacia Gene Assembler synthesizer according to the manufacturer's recommendations using phosphoramidite chemistry. The purified oligonucleotides were annealed, phosphorylated and cloned by blunt end ligation into the filled in *Sal*I site of the Bsp methyltransferase (Bsp Mase) gene (Pósfai et al., 1986). Stop codons in all three reading frames present in the noncoding strand of the NLS oligonucleotide ensure that ligation of the NLS oligonucleotide in the antisense orientation will generate a 38 kDa truncated fusion protein, while the incorporation of 1 or 2 copies of the NLS oligo in the sense orientation will generate a 46 kDa, or 48 kDa long fusion protein, respectively. After transformation into an *E. coli* HB 101 strain carrying the compatible pVH1 plasmid which contains the *lac* I^q gene and a kanamycin resistance marker, transformants were selected on kanamycin-ampicillin plates. Recombinants carrying the NLS oligo were selected by colony hybridization with the end labeled NLS oligonucleotide as probe. 2 ml cultures from the selected recombinant colonies were grown until an A_{550}=0.5, the cultures were split, one half was grown for an additional 3 hours without induction, the other half was induced with 10 mM IPTG. Cellular proteins were analyzed on 10% SDS-polyacrylamide gel (Fig. 1B). Recombinants carrying 0, 1 or 2 copies of the NLS (Bsp Mase, Bsp Mase-NLS_1 or Bsp Mase-NLS_2) were selected, and the DNA sequence in the cloned region was confirmed by sequencing.

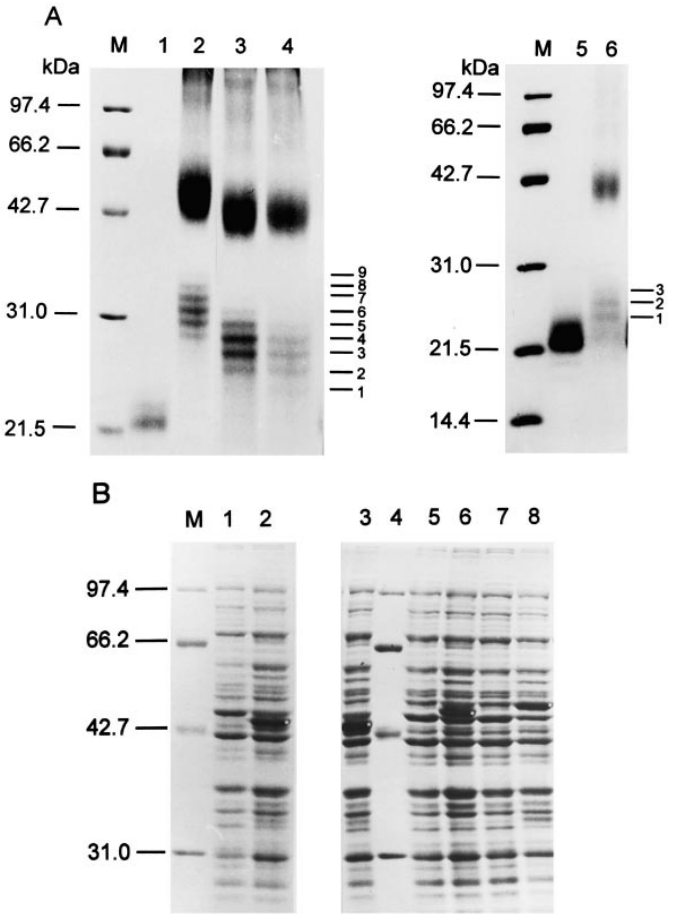


Fig. 1. SDS-polyacrylamide gel pattern of the nuclear transport substrates. (A) PE-NLS conjugates were separated on 13% SDS-polyacrylamide gels and visualized by Coomassie Blue staining. The size of the marker proteins in lanes M are indicated on the left side; the number of conjugated NLSs is marked on the right side of each gel. Lane 1, unconjugated PE; lane 2, PE-$NLS_{SMCC7\text{-}8}$; lane 3, PE-$NLS_{SMCC4\text{-}5}$; lane 4, PE-$NLS_{MBS4\text{-}5}$; lane 5, unconjugated PE; lane 6, PE-$NLS_{MBS1\text{-}2}$. (B) Total cell protein of uninduced and induced cultures of *E. coli* carrying the Bsp Mase-NLS fusion genes were separated on a 10% SDS-polyacrylamide gel and stained by Coomassie Blue. The size of the marker proteins in lanes M and 4 is indicated on the left side. Uninduced (lane 1) and induced (lanes 2 and 3) cultures of *E. coli* carrying the Bsp Mase gene without NLS; uninduced (lane 5) and induced (lane 6) cultures of *E. coli* carrying the Bsp Mase-NLS_1 gene; uninduced (lane 7) and induced (lane 8) cultures of *E. coli* carrying the Bsp Mase-NLS_2 gene. The induced fusion proteins are marked by asterisk.

Bsp Mase-NLS fusion proteins were purified from 6 litre cultures after inducing the cells at an A_{550}=0.5 with 0.1% lactose. The cells were grown for an additional 4 hours, centrifuged, washed with 0.15 M NaCl and resuspended into 3 vols of sonication buffer (0.3 M NaCl, 20 mM Tris-Cl, pH 7.6, 5 mM beta-mercaptoethanol and 5% glycerol). Cells were disrupted by sonication (3 minutes with full power in a Branson B 30 sonifier) and centrifuged for an hour at 110,000 ***g*** in a Beckman Ti 60 rotor. Nucleic acids were removed by polyethylenimine precipitation. By mixing a small aliquot of the extract with increasing concentrations of polyethyleneimine, the optimum precipitation condition which quantitatively removes the nucleic acids, was determined. Proteins from the nucleic acid free extract were precipitated by ammonium sulfate to 80% saturation, the pellet was dissolved in 40 ml of sonication buffer, and loaded without dialysis onto a 2 × 18 cm hydroxylapatite column (Bio-Rad) equilibrated with 25 mM K phosphate, pH 6.7, 5 mM beta-mercaptoethanol and 5% glycerol. After thorough washing of the column with this buffer, proteins were eluted with a linear K phosphate gradient (25-400 mM). Gradient fractions were tested by immuno-dot-blot analysis, using a polyclonal antibody specific for Bsp methyltransferase. Bsp Mase-NLS fusion proteins elute between 300-400 mM K phosphate. These fractions were dialysed against 50 mM NaCl, 20 mM Tris-Cl, pH 7.6, 5 mM beta-mercaptoethanol and 5% glycerol, and further purified by ion-exchange FPLC chromatography on a Poros 20 HQ column (PerSeptive Biosystems). Proteins were eluted by a linear NaCl gradient (50-400 mM). Bsp Mase-NLS fusion proteins were 95-98% pure in the peak fraction. The purified proteins were mixed with an equal volume of 1 mg/ml fluorescein isothiocyanate-celite (FITC-celite, Sigma) dissolved in 0.5 M sodium carbonate buffer, pH 9.0, containing 0.1 M NaCl, and incubated at room temperature for 2 hours on a rocking platform. The FITC-Bsp Mase-NLS conjugate was finally purified on a Sephadex G25 column equilibrated with 0.1 M NaCl, 20 mM Hepes, pH 7.3, and 1 mM EDTA, concentrated in a Centricon 10 microconcentrator to 0.25 mg/ml and stored in small aliquots at –80°C.

In vitro nuclear transport in digitonin-permeabilized cells

HeLa cells were grown in Ham's F-12 nutrient mixture (Gibco-BRL) supplemented with 5% fetal calf serum. Subconfluent cultures of the cell lines grown on coverslips were permeabilized with digitonin as described by Adam et al. (1990). Digitonin obtained from two different suppliers (Sigma and Calbiochem) were tested. The digitonin was dissolved in dimethylsulfoxide at a concentration of 20 mg/ml and freshly diluted to 40 μg/ml in transport buffer (Adam et al., 1990). Permeabilization was performed at 0°C for 5 minutes, followed by thorough washing in transport buffer at 0°C. The coverslips were then blotted to remove excess liquid, placed on a 50 μl drop of transport mixture or complete transport mixture, and incubated for 30 minutes at the indicated temperature in a humidified chamber. The transport mixture contained 1 mg/ml BSA and 1 μg of NLS-conjugated transport substrate dissolved in transport buffer. The complete transport mixture was the transport mixture supplemented with 1 mM ATP (Sigma), 5 mM creatine phosphate (Sigma) and 20 U/ml creatine phosphokinase (Sigma). After this incubation, the coverslips were thoroughly washed in transport buffer, fixed in 4% formaldehyde (diluted in transport buffer) for 10 minutes at room temperature,

washed briefly in phosphate-buffered saline and mounted on a drop of 1 mg/ml *p*-phenylenediamine (Aldrich) in 10% phosphate-buffered saline and 90% glycerol. The edges of the coverslip were sealed with nail polish and the cells were observed and photographed on an Olympus Vanox-S AH2 microscope equipped with SPlan 10NH and DPlan Apo100 UVPL objectives. Comparative experiments were carried out on cells grown on coverslips incubated in the same tissue culture dish and photographed for the same length of time.

To study the effect of cytosolic protein depletion on the nuclear transport, the basic permeabilization protocol described above (Adam et al., 1990) was modified by increasing the concentration of digitonin and/or the duration of permeabilization as indicated. Cytosol from HeLa cells was prepared as described by Adam et al. (1990). *Drosophila* embryonic extract was prepared as described earlier (Udvardy, 1993). The effect of cytosolic proteins on the transport reaction was tested by supplementing the complete transport mixture with increasing amounts of cytosolic extracts.

To study the effect of WGA on the in vitro nuclear transport reaction, permeabilized HeLa cells were preincubated on a 50 µl drop of transport buffer supplemented with 1 mg/ml BSA and 200 µg/ml WGA (Sigma) for 10 minutes at 0°C. The cells were washed in transport buffer, further incubated on a 50 µl drop of complete transport mixture at 30°C for 30 minutes, washed and fixed as described above.

The integrity of the nuclear membrane of digitonin permeabilized cells was tested with a human autoimmune serum containing anti-DNA antibodies (Hadlaczky and Praznovszky, 1988). HeLa cells permeabilized for 5, 10 or 15 minutes in 40, 60 or 80 µg/ml digitonin, were incubated in a complete transport mixture containing MBS conjugated PE-NLS as transport substrate and the autoimmune serum (1:200 dilution) for 30 minutes at 30°C. After thorough washing of the cells in transport buffer, the incubation was continued at room temperature for 15 minutes in transport buffer supplemented with FITC labelled immunoglobulin to human IgG (Hyland). The coverslips were washed three times in PBS, incubated for 10 minutes at room temperature in PBS containing 4% formaldehyde, mounted and photographed using the built in FITC or TRITC filters of the Olympus Vanox-S AH2 microscope. In control experiments, no staining was observed when only the FITC labelled second antibody was used. The reactivity of the autoimmune serum with the nuclear DNA was tested on cells incubated in PBS containing 0.2% gelatin and 0.2% Triton X-100 (Sigma) for 6 minutes at room temperature. After washing of the cells in PBS + 0.2% gelatin, the immunostaining was performed as described above.

To measure the amount of protein solubilized by digitonin permeabilization, HeLa cells were grown overnight in Ham's F 12 medium containing half the normal concentration of methionine and supplemented with 5% fetal calf serum and 5 uCi/ml [^{35}S]methionine. Before permeabilization, the cells were labeled for an additional 3 hours in methionine-free medium supplemented with 5% fetal calf serum and 20 uCi/ml [^{35}S]methionine. The cells were rinsed three times with transport buffer and permeabilized at 0°C. The concentration of digitonin and the duration of the permeabilization were varied as indicated. Proteins released from the cells during the permeabilization were removed, and the radioactivity of an aliquot of the extracted proteins was measured in a liquid scintillation cocktail. The permeabilized cells were scraped from the plate with a rubber policeman and suspended in a measured volume of buffer, and aliquots were counted in liquid scintillation cocktail.

RESULTS

Nuclear transport of PE-NLS$_{MBS}$

PE-NLS$_{MBS}$ transport substrate was prepared by chemically conjugating the SV 40 large T antigen NLS to PE, a high molecular mass (132 kDa), naturally fluorescent protein by the aid of the heterobifunctional cross-linker $_{MBS}$. The NLS in this transport substrate is attached to PE through a maleimidobenzoyl residue (Fig. 2). If the low molecular mass impurities derived from the hydrolysis products of the chemical cross-linker were completely removed by an appropriate purification step (see Materials and Methods), this transport substrate was efficiently transported to the nucleus of digitonin-permeabilized cells without any exogenously added cytosolic protein (Fig. 3).

The nuclear transport of the highly purified PE-NLS$_{MBS}$ conjugate in the absence of any exogenously added protein fulfils all the criteria of a selective, mediated transport: (i) due to its large size (132 kDa), PE alone, without conjugated NLS, cannot enter the nucleus by passive diffusion (Fig. 3A), and its transport is not achieved by the addition of either a *Drosophila* embryonic extract or HeLa cytosol (data not shown). (ii) The nuclear transport of the PE-NLS$_{MBS}$ conjugate is absolutely energy dependent. The nuclear import was blocked if the transport reaction was performed at 0°C in the absence of ATP (Fig. 3B). If the transport reaction was incubated at 30°C in the presence of ATP and an ATP regenerating system, there was a highly efficient nuclear transport, the cytoplasmic fluorescence completely disappeared, and the nuclei exhibited a bright fluorescence (Fig. 3E). The addition of a *Drosophila* embryonic extract or a HeLa cytosol did not enhance this transport reaction (data not shown). If the transport reaction was incubated at 30°C without exogenously added ATP, there was a certain level of nuclear accumulation of the transport substrate, although a large fraction of the substrate remained in the cytoplasm (Fig. 3C). In order to demonstrate that this

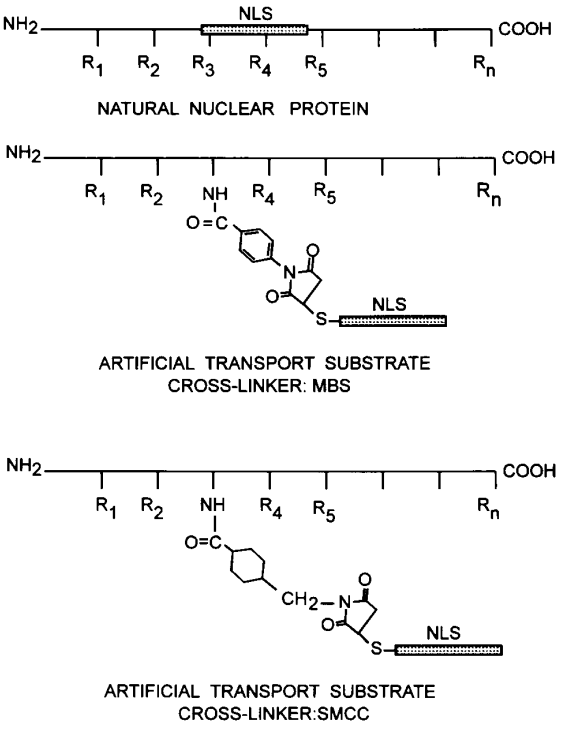


Fig. 2. Schematic structure of natural and artificial transport substrates.

transport is due to residual ATP produced in digitonin-permeabilized cells, HeLa cells permeabilized under standard conditions were preincubated on a 50 μl drop of transport buffer supplemented with 1 mM $CaCl_2$, 1 mg/ml BSA and 1 unit of apyrase for 5 minutes at 30°C, and the coverslip was then placed on a new 50 μl drop of transport buffer supplemented with 1 mM $CaCl_2$, 1 mg/ml BSA, 1 unit of apyrase and 1 μg of PE-NLS_{MBS}, and incubated for an additional 30 minutes at 30°C. As shown in Fig. 3D, apyrase treatment greatly reduced the nuclear and especially the nucleolar accumulation of the transport substrate. The perinuclear accumulation of the transport substrate, however, was not affected. The calcium in the above reaction was required by the apyrase, and it does not influence the transport reaction (data not shown). (iii) The transport of PE-NLS_{MBS} conjugate observed in digitonin-permeabilized cells without exogenously added cytosolic proteins is dependent on the presence of a transport-competent NLS. Only a weak nucleolar accumulation was observed if a

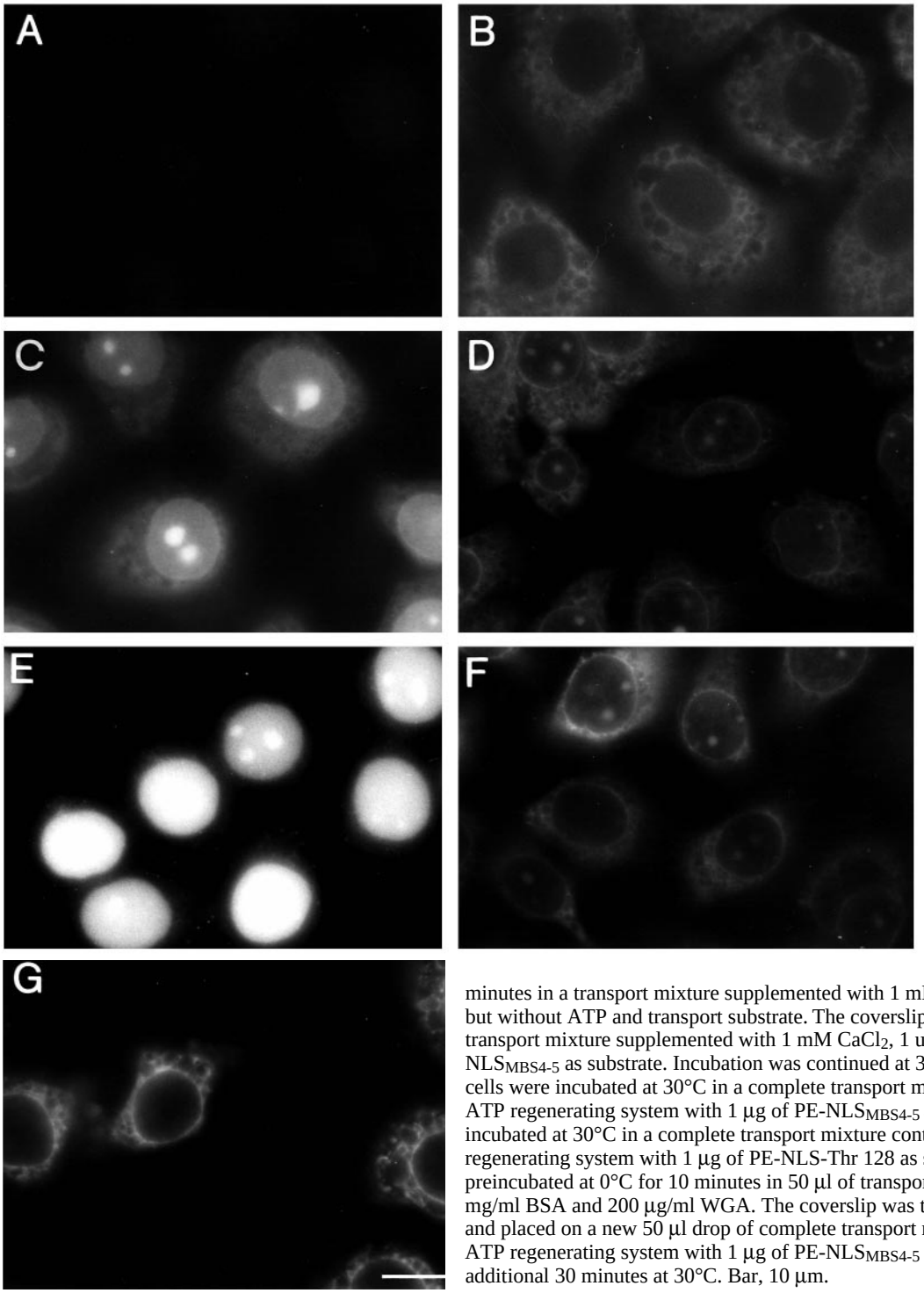


Fig. 3. Specificity of the nuclear transport in digitonin-permeabilized HeLa cells. PE-NLS_{MBS4-5} was used as substrate. HeLa cells were permeabilized in 40 μg/ml digitonin for 5 minutes and the transport reaction was incubated for 30 minutes at the temperature indicated. (A) HeLa cells were incubated at 30°C in a complete transport mixture containing ATP and an ATP regenerating system with unconjugated PE as substrate. (B) HeLa cells were incubated at 0°C in a transport mixture containing 1 μg of PE-NLS_{MBS4-5} as substrate without ATP and an ATP regenerating system. (C) HeLa cells were incubated at 30°C in a transport mixture containing 1 μg of PE-NLS_{MBS4-5} as substrate without ATP and an ATP regenerating system. (D) HeLa cells were preincubated at 30°C for 5 minutes in a transport mixture supplemented with 1 mM $CaCl_2$ and 1 unit of apyrase, but without ATP and transport substrate. The coverslip was then placed on a new drop of transport mixture supplemented with 1 mM $CaCl_2$, 1 unit of apyrase and 1 μg of PE-NLS_{MBS4-5} as substrate. Incubation was continued at 30°C for 30 minutes. (E) HeLa cells were incubated at 30°C in a complete transport mixture containing ATP and an ATP regenerating system with 1 μg of PE-NLS_{MBS4-5} as substrate. (F) HeLa cells were incubated at 30°C in a complete transport mixture containing ATP and an ATP regenerating system with 1 μg of PE-NLS-Thr 128 as substrate. (G) HeLa cells were preincubated at 0°C for 10 minutes in 50 μl of transport buffer supplemented with 1 mg/ml BSA and 200 μg/ml WGA. The coverslip was then washed in transport buffer and placed on a new 50 μl drop of complete transport mixture containing ATP and an ATP regenerating system with 1 μg of PE-NLS_{MBS4-5} as substrate and incubated for an additional 30 minutes at 30°C. Bar, 10 μm.

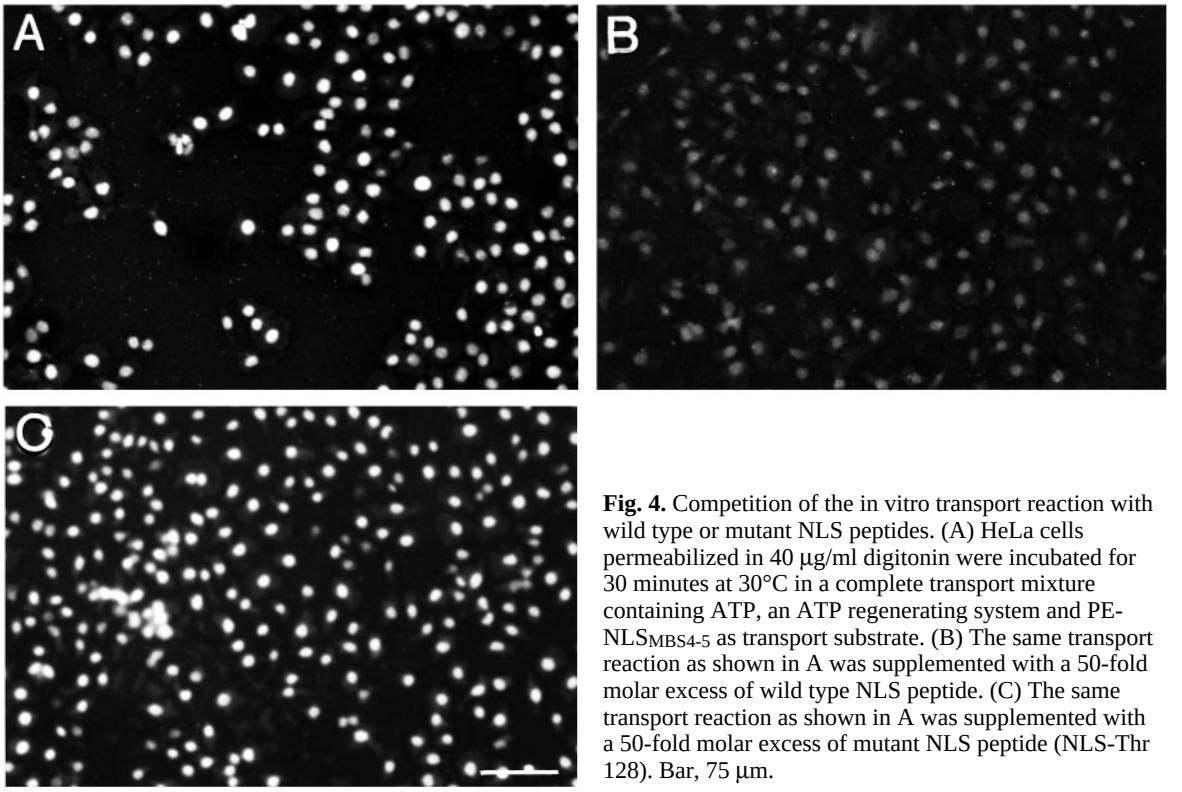


Fig. 4. Competition of the in vitro transport reaction with wild type or mutant NLS peptides. (A) HeLa cells permeabilized in 40 μg/ml digitonin were incubated for 30 minutes at 30°C in a complete transport mixture containing ATP, an ATP regenerating system and PE-NLS_{MBS4-5} as transport substrate. (B) The same transport reaction as shown in A was supplemented with a 50-fold molar excess of wild type NLS peptide. (C) The same transport reaction as shown in A was supplemented with a 50-fold molar excess of mutant NLS peptide (NLS-Thr 128). Bar, 75 μm.

transport-incompetent mutant NLS (NLS-Thr 128) was conjugated to PE (Fig. 3F). Some transport by the mutant NLS is not unexpected, since it can function as a weak NLS (Goldfarb et al., 1986; Newmeyer and Forbes, 1988; Lanford et al., 1986). (iv) The transport is totally inhibited by lectin WGA (Fig. 3G). WGA, however, did not influence the perinuclear accumulation of the transport substrate. (v) The transport machinery in digitonin-permeabilized cells is saturable, the nuclear accumulation of PE-NLS_{MBS} can be efficiently competed by the addition of a 50-fold molar excess of wild type NLS peptide, whereas the transport-incompetent mutant NLS-Thr 128 peptide did not compete for the transport in this concentration (Fig. 4B,C).

The level of nuclear accumulation of the PE conjugates in a digitonin-permeabilized cell was proportional to the number of conjugated NLS sequences. As shown in Fig. 5A, the nuclear accumulation of PE-NLS_{MBS1-2}, a transport substrate with low conjugation level (1-2 NLSs/subunit), was much lower than that of PE-NLS_{MBS4-5} (4-5 NLSs/subunit) (Fig. 5B).

To prove that the nuclear accumulation of the PE-NLS_{MBS} conjugate in digitonin-permeabilized HeLa cells without any exogenously added cytosolic protein was not due to perme-

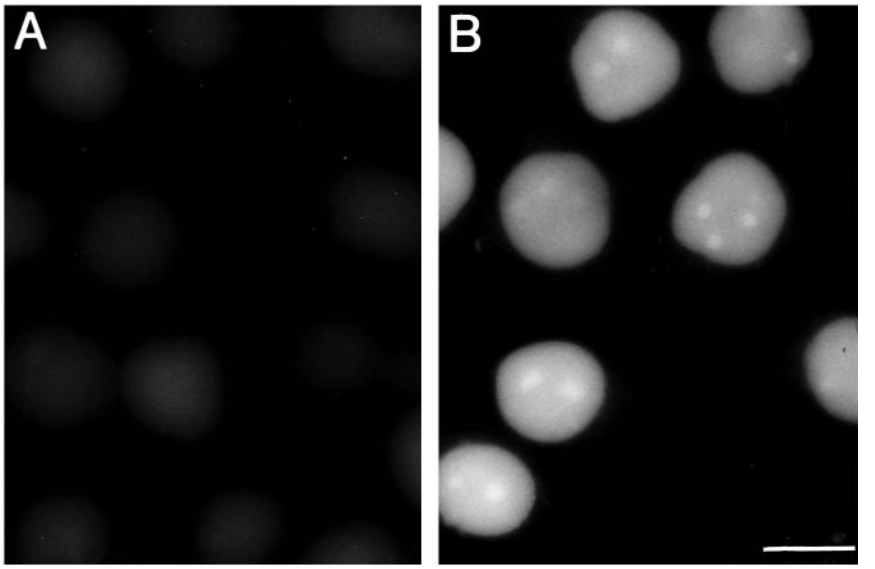


Fig. 5. Effect of the number of conjugated NLS peptides on the transport efficiency. HeLa cells permeabilized in 40 μg/ml digitonin were incubated at 30°C in a complete transport mixture containing ATP, an ATP regenerating system and 1 μg of transport substrate. (A) PE-NLS_{MBS1-2} as transport substrate. (B) PE-NLS_{MBS4-5} as transport substrate. Bar, 10 μm.

abilization of the nuclear membrane, HeLa cells were permeabilized with increasing concentrations of digitonin, and the transport experiments were repeated with PE-NLS_{MBS4-5} as substrate in the presence of a human autoimmune serum containing anti-DNA IgG. Indirect immunofluorescence with a FITC labelled anti-human IgG permitted the simultaneous localization of the anti-DNA antibody and the transport substrate in the same permeabilized cells. The nuclear accumulation of PE-NLS_{MBS} was not impaired by increasing the digitonin concentration during the permeabilization from 40 μg/ml (Adam et al., 1990) up to 80 μg/ml (Fig. 6A and C). The nuclear DNA, however, was inaccessible for the anti-DNA antibody (Fig. 6A-D). The nuclear DNA was readily accessible for the anti-DNA antibody after removal of the outer nuclear membrane by Triton X-100 treatment (Fig. 6E). As detected by the exclusion of the anti-DNA antibody, the nuclear membrane was not damaged in the digitonin concentration range 40 to 80 μg/ml (Fig. 6B and D).

To prove that the nuclear transport of highly purified PE-NLS_{MBS} without any exogenously added protein is not due to incomplete extraction of cytosolic proteins from the digitonin permeabilized cells, the fraction of HeLa cell proteins extracted during the permeabilization was measured as a function of digitonin concentration and permeabilization time.

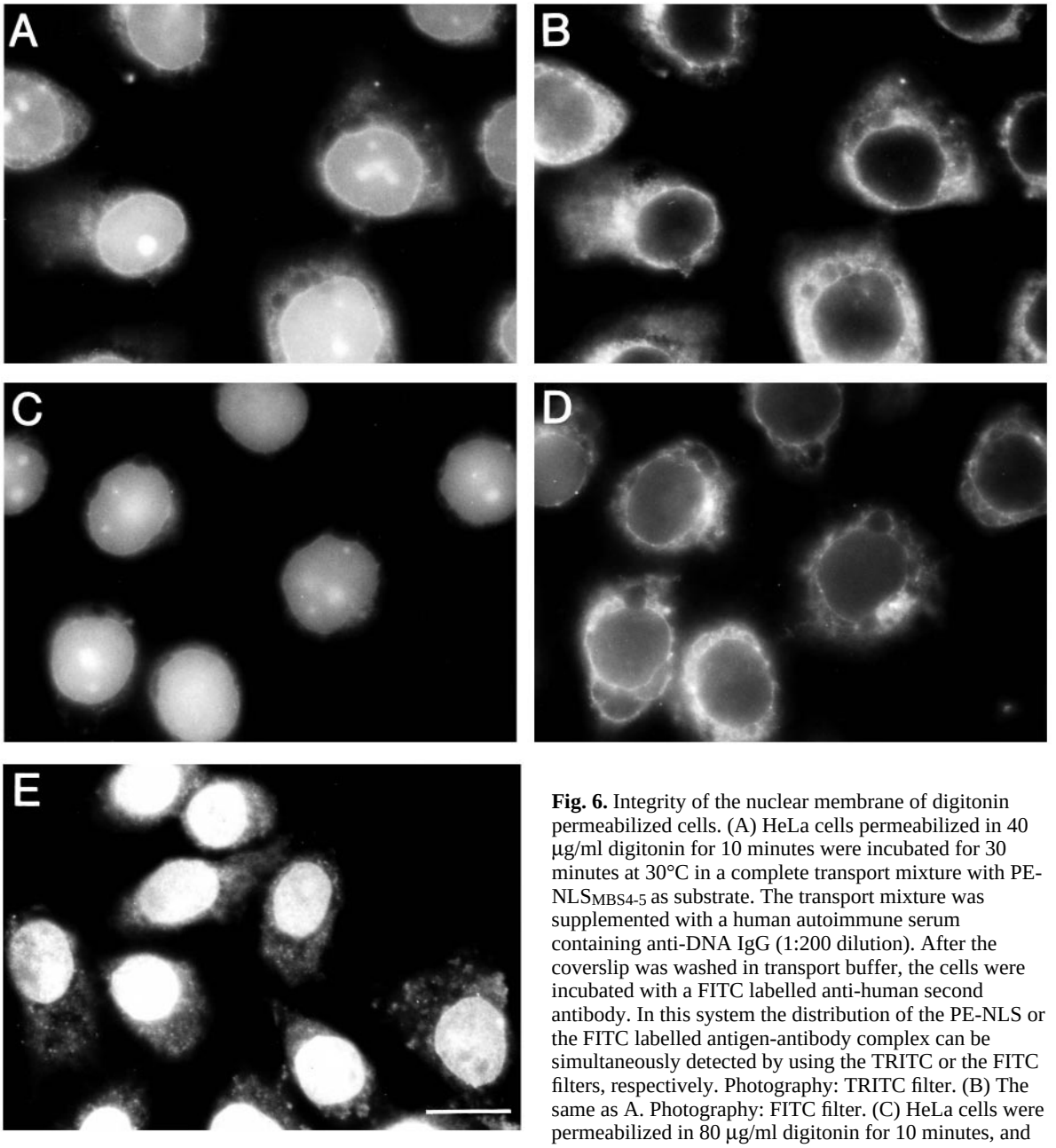


Fig. 6. Integrity of the nuclear membrane of digitonin permeabilized cells. (A) HeLa cells permeabilized in 40 μg/ml digitonin for 10 minutes were incubated for 30 minutes at 30°C in a complete transport mixture with PE-NLS_{MBS4-5} as substrate. The transport mixture was supplemented with a human autoimmune serum containing anti-DNA IgG (1:200 dilution). After the coverslip was washed in transport buffer, the cells were incubated with a FITC labelled anti-human second antibody. In this system the distribution of the PE-NLS or the FITC labelled antigen-antibody complex can be simultaneously detected by using the TRITC or the FITC filters, respectively. Photography: TRITC filter. (B) The same as A. Photography: FITC filter. (C) HeLa cells were permeabilized in 80 μg/ml digitonin for 10 minutes, and the nuclear transport was tested under the conditions described in A. Photography: TRITC filter. (D) The same as C. Photography: FITC filter. (E) Reactivity of human autoimmune serum with nuclear DNA. Triton X-100 treated HeLa cells were incubated with human autoimmune serum (1:200 dilution) for 15 minutes at room temperature, thoroughly washed and stained with FITC labelled anti-human second antibody. Photography: FITC filter. Bar, 10 μm.

At 40 μg/ml digitonin, 17% of the total cellular protein was extracted during permeabilization for 5 minutes (Table 1). This is in good agreement with previously published data (Adam et al., 1990), indicating that the nuclear transport observed is not due to incomplete extraction of cytosolic proteins. The total amount of protein extracted from permeabilized cells did not increase significantly on increase of the digitonin concentration from 40 to 80 μg/ml (Table 1). Permeabilization for 5 minutes, however, is not sufficient for the complete release of soluble cytosolic proteins. On extension of the permeabilization time, the quantity of cytosolic proteins released increased at all digitonin concentrations tested, and reached a plateau after permeabilization for 15 minutes There was only a slight decline in the nuclear transport ability of HeLa cells permeabilized for 5 or 10 minutes in 40 or 80 μg/ml digitonin (Fig. 7A,B,D,E). The nuclear transport, however, was significantly reduced after permeabilization for 15 minutes, especially in 80 μg/ml digitonin (Fig. 7C and F). The integrity of the nuclear membrane, as detected by the exclusion of the anti-DNA antibody, was not impaired during the first 10 minutes of permeabilization (Fig. 6B and D). The nuclear membrane, however, was damaged in a fraction of cells after longer permeabilization. Thus, in subconfluent cultures of HeLa cells permeabilization for 15 minutes in 40 or 80 μg/ml digitonin damaged the nuclear membrane in 5-10 %, or 15-20% of the cells, respectively (data not shown). These figures were even higher in confluent, stationary cultures.

Nuclear transport of PE-NLS$_{SMCC}$

PE-NLS$_{SMCC}$ transport substrate was prepared by chemically conjugating the SV 40 large T antigen NLS to PE by the heterobifunctional cross-linker SMCC. The NLS in this transport substrate is attached to PE through a (*N*-maleimidomethyl)cyclohexane-1-carboxylate residue (Fig. 2). The maximal conjugation level achieved with this cross-linker was 7-8 NLSs/subunit. This high level of conjugation has never been achieved by MBS (maximal conjugation was 4-5 NLSs/subunit). Although comparing the nuclear accumulation of PE-NLS$_{SMCC4-5}$ and PE-NLS$_{MBS4-5}$ in digitonin permeabilized cells by densitometric scanning of photographic negatives revealed that PE-NLS$_{SMCC4-5}$ is about a 20-25% less efficient transport substrate, its transport was also cytosol independent (Fig. 8A). This was in sharp contrast with the nuclear transport of the highly conjugated PE-NLS$_{SMCC7-8}$, which was absolutely dependent on the addition of exogenous cytosolic proteins. In a cytosol-free transport reaction, only a very weak nucleolar accumulation was observed, while the majority of the substrate was in the cytoplasm (Fig. 8B). Exogenously added cytosol completely reversed this distribution. In the presence of cytosol, nuclei showed a bright fluorescence, while the transport substrate is completely removed from the cytoplasm (Fig. 8C). Further, a *Drosophila* embryonic extract was shown to be more effective, supporting this transport in comparison with a HeLa cytosol (data not shown). All the characteristic features of the in vitro nuclear transport reaction described by Adam et al. (1990) and Shannon Moore and Blobel (1992) were faithfully reproduced with this transport substrate: the reaction was ATP dependent, cytosolic proteins without ATP supported only the binding of the substrate to the nuclear envelope (Fig. 8D), the translocation step required ATP. The transport was blocked by WGA, and it was dependent on the presence of a functional wild type NLS (data not shown). Similarly, the cytosol independent transport of PE-NLS$_{SMCC1-2}$ and PE-NLS$_{SMCC4-5}$ was energy-dependent, it was blocked by WGA and required the presence of a functional wild type NLS (data not shown).

Nuclear transport of Bsp Mase-NLS fusion protein

To study the mechanism of nuclear transport of a protein in which the same NLS, present in the above described artificial transport substrates is incorporated in a natural context (i.e. as a segment of the peptide backbone linked through peptide bonds), an oligonucleotide coding for the SV40 large T antigen NLS was cloned in one, or two, copies into the unique *Sal*I site present in the gene of the bacterial enzyme Bsp methyltransferase. The expressed and purified fusion proteins were FITC labelled and tested for nuclear transport in digitonin-permeabilized HeLa cells. The 44 kDa Bsp Mase (without a fused NLS segment) was not transported at 30°C in the presence of ATP, and neither HeLa cytosol, nor *Drosophila* embryonic extract could promote this transport (data not shown). Bsp Mase with a single copy of NLS (Bsp Mase-NLS$_1$) was transported at a low level (data not shown). Bsp Mase with two copies of NLS (Bsp Mase-NLS$_2$) was efficiently transported at 30°C in the presence of ATP without any exogenous cytosol (Fig. 8E), and neither HeLa cytosol, nor *Drosophila* embryonic extract improved this transport (data not shown). This transport was absolutely energy dependent (Fig. 8F), and it was blocked by WGA (Fig. 8G).

Distinct transport mechanisms operate in digitonin-permeabilized cells

The cytosol independent transport of Bsp Mase-NLS (or the artificial transport substrates with low NLS conjugation level) may either be the consequence of an inefficient extraction of cytosolic proteins during digitonin permeabilization, or it may represent a distinct transport mechanism which cannot operate on multiconjugated transport substrates. To test these alternatives the simultaneous transport of PE-NLS$_{SMCC7-8}$ and Bsp Mase-NLS$_2$ was studied in digitonin-permeabilized cell. In these experiments the complete transport mixture was supplemented with 0.75 μg of PE-NLS$_{SMCC7-8}$ and 0.25 μg of Bsp Mase-NLS$_2$, and the simultaneous transport of these substrates was followed by photography using the FITC or TRITC filters. As shown in Fig. 8H and I, in the absence of cytosol Bsp Mase-NLS$_2$ was efficiently transported, while PE-NLS$_{SMCC7-8}$ was retained in the cytoplasm. In the presence of cytosol, however, PE-NLS$_{SMCC7-8}$ was just as efficiently transported as Bsp Mase-NLS$_2$ (data not shown). PE-NLS$_{SMCC4-5}$ and Bsp Mase-

Table 1. Release of soluble proteins during digitonin permeabilization

	Percentage of total protein released		
	40 μg/ml Digitonin	60 μg/ml Digitonin	80 μg/ml Digitonin
5 Min	17.1	18.4	19.8
10 Min	26.8	30.2	32.6
15 Min	34.3	36.5	38.0
20 Min	36.1	38.7	39.9

Average of three experiments.

NLS_2 (Fig. 8J and K), or PE-NLS_{MBS4-5} and Bsp Mase-NLS_2 (data not shown) were simultaneously transported in the absence of cytosol.

DISCUSSION

The transport of proteins from the cytoplasm to the nucleus is a complex, multistep process. Recognition of the NLS-containing protein by cytoplasmic receptor(s) is presumed to be the first step of a mediated nuclear transport. The next step is suggested to be targeting of this protein to the nuclear envelope by the receptor protein. These steps, which were shown to be energy independent are followed by an energy dependent translocation step through the nuclear pore.

Recently, two different in vitro nuclear transport systems were developed to characterize the soluble cytosolic proteins involved in this transport process (Newmeyer and Forbes, 1988; Adam et al., 1990). Due to its versatility and efficiency, in vitro nuclear transport in digitonin-permeabilized tissue culture cells has gained widespread application (Adam and Gerace, 1991; Stochaj and Silver, 1992; Sterne-Marr et al.,

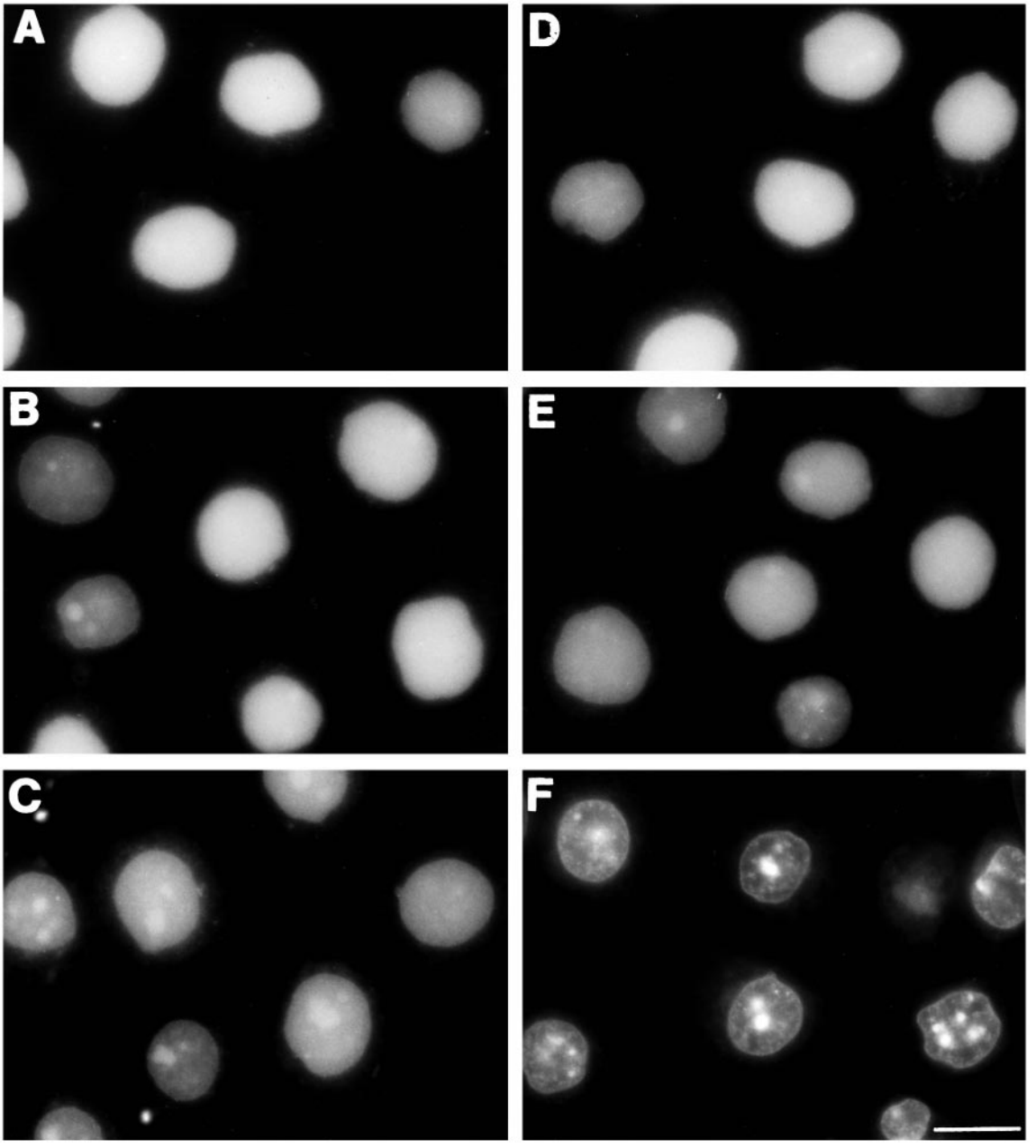


Fig. 7. Extensive removal of cytosolic proteins during digitonin permeabilization does not impair the transport ability of HeLa cells. HeLa cells permeabilized at 0°C under the conditions described below were incubated in a complete transport mixture with PE-NLS_{MBS4-5} as substrate at 30°C for 30 minutes. (A) Permeabilization in 40 μg/ml digitonin for 5 minutes. (B) Permeabilization in 40 μg/ml digitonin for 10 minutes. (C) Permeabilization in 40 μg/ml digitonin for 15 minutes. (D) Permeabilization in 80 μg/ml digitonin for 5 minutes. (E). Permeabilization in 80 μg/ml digitonin for 10 minutes. (F) Permeabilization in 80 μg/ml digitonin for 15 minutes. Bar, 10 μm.

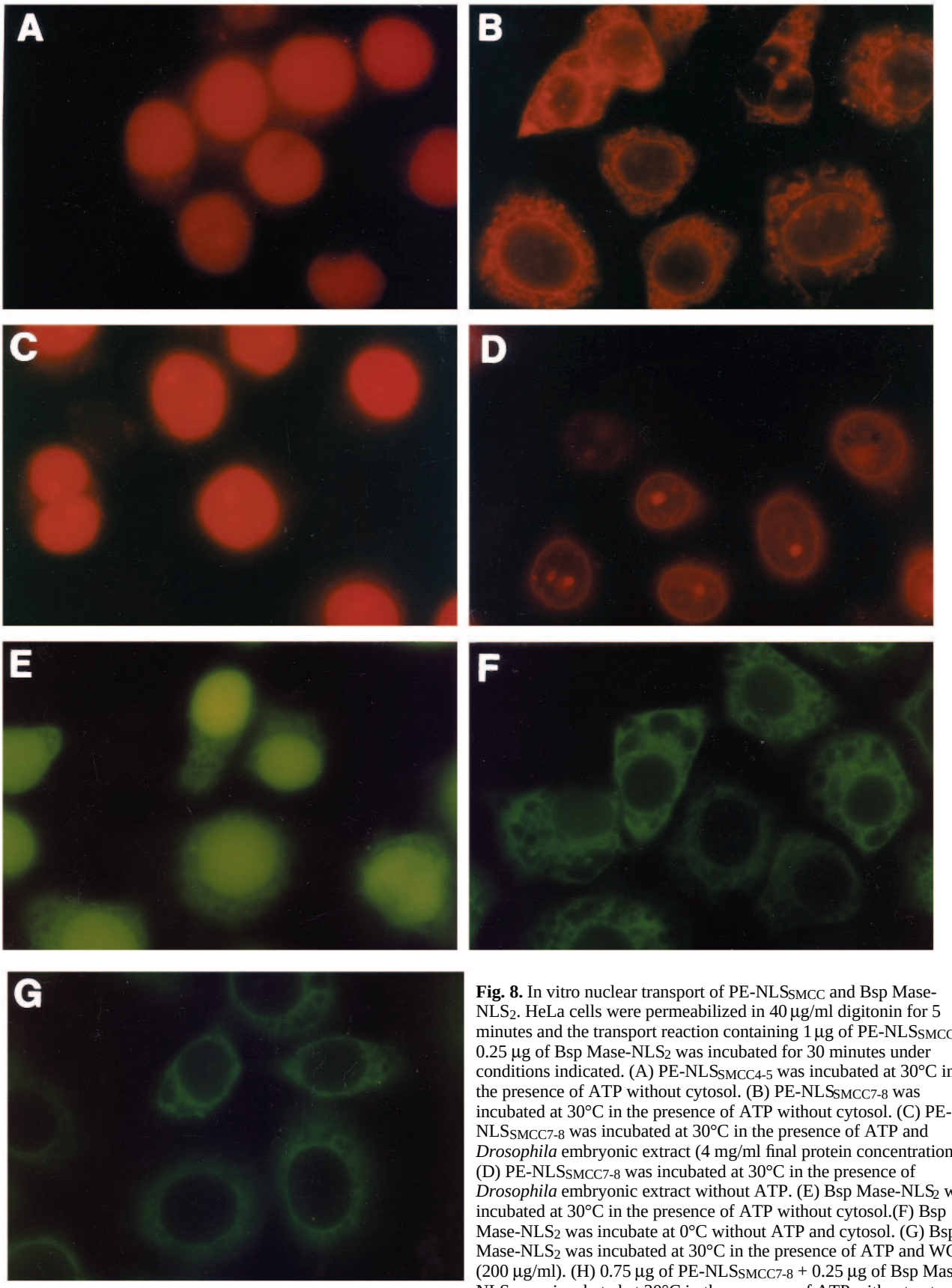


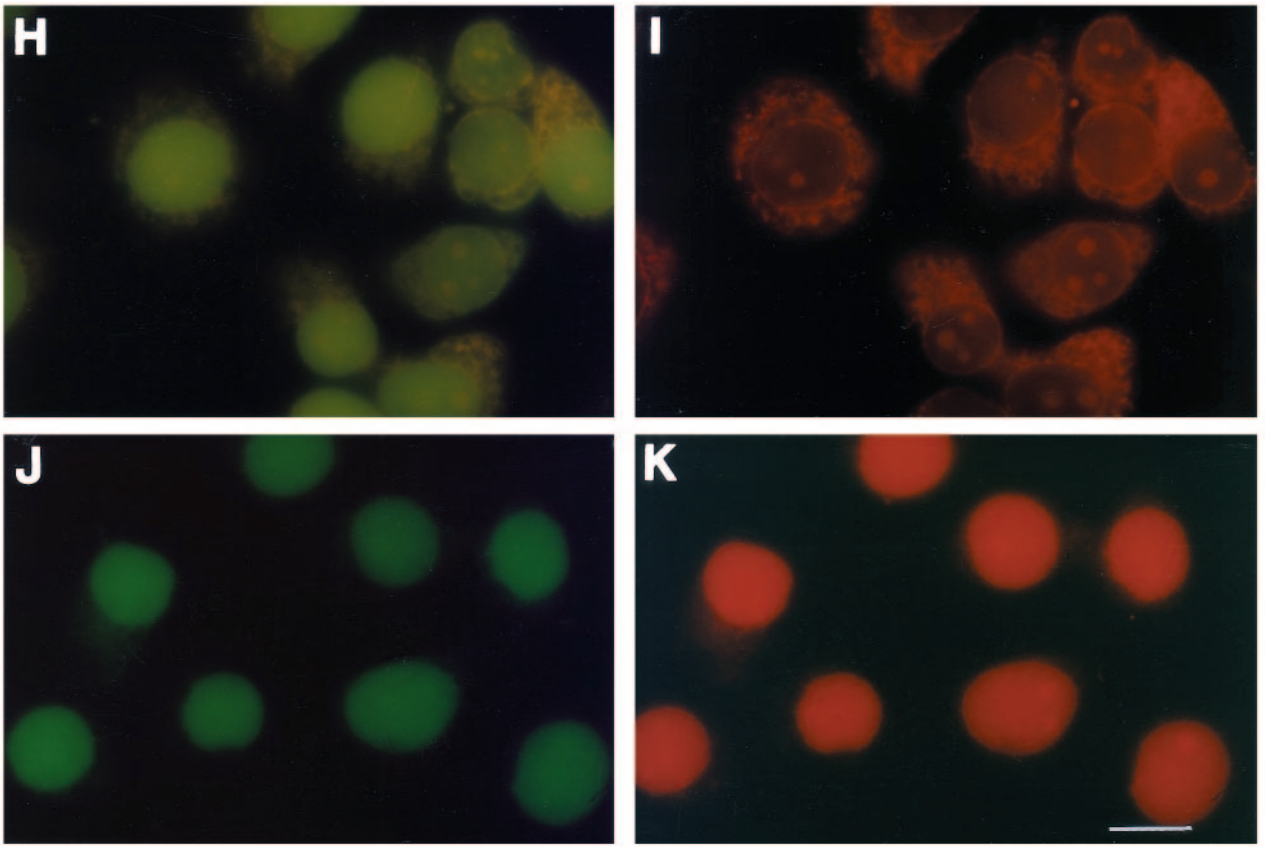
Fig. 8. In vitro nuclear transport of PE-NLS$_{SMCC}$ and Bsp Mase-NLS$_2$. HeLa cells were permeabilized in 40 μg/ml digitonin for 5 minutes and the transport reaction containing 1 μg of PE-NLS$_{SMCC}$ or 0.25 μg of Bsp Mase-NLS$_2$ was incubated for 30 minutes under conditions indicated. (A) PE-NLS$_{SMCC4-5}$ was incubated at 30°C in the presence of ATP without cytosol. (B) PE-NLS$_{SMCC7-8}$ was incubated at 30°C in the presence of ATP without cytosol. (C) PE-NLS$_{SMCC7-8}$ was incubated at 30°C in the presence of ATP and *Drosophila* embryonic extract (4 mg/ml final protein concentration). (D) PE-NLS$_{SMCC7-8}$ was incubated at 30°C in the presence of *Drosophila* embryonic extract without ATP. (E) Bsp Mase-NLS$_2$ was incubated at 30°C in the presence of ATP without cytosol.(F) Bsp Mase-NLS$_2$ was incubate at 0°C without ATP and cytosol. (G) Bsp Mase-NLS$_2$ was incubated at 30°C in the presence of ATP and WGA (200 μg/ml). (H) 0.75 μg of PE-NLS$_{SMCC7-8}$ + 0.25 μg of Bsp Mase-NLS$_2$ was incubated at 30°C in the presence of ATP without cytosol. Photography: FITC filter. (I) Same as H. Photography: RITC filter. (J) 0.75 μg of PE-NLS$_{SMCC4-5}$ + 0.25 μg of Bsp Mase-NLS$_2$ was incubated at 30°C in the presence of ATP without cytosol. Photography: FITC filter. (K) Same as J. Photography: TRITC filter. Bar, 10 μm.

1992; Shannon Moore and Blobel, 1992; Shi and Thomas, 1992; Hennekes et al., 1993). Nuclear transport in digitonin-permeabilized tissue culture cells has been reported to require exogenously added cytosolic proteins. A wide variety of heterologous cell lysates supported the nuclear import in HeLa and NRK cells (Adam et al., 1990). Three different proteins, involved at different steps of the transport process have been purified and characterized. NLS-mediated binding to the nuclear envelope required the cytosolic NLS receptor (Adam and Gerace, 1991) and a 97 kDa protein (Adam and Adam, 1994), while the translocation step was dependent on the Ran/TC4 GTP-binding protein (Shannon Moore and Blobel, 1993). In these experiments, artificial transport substrates served to follow the nuclear transport reaction. The number and the conformation of the NLS in an artificial nuclear transport substrate or in a natural nuclear protein is basically different. The NLS in a natural nuclear protein is part of the polypeptide backbone, attached to the rest of the protein through natural peptide bonds. In the artificial nuclear transport substrate the NLS is attached to the protein through a nonnatural cyclic compound derived from the cross-linker, and with the exception of the N-terminal free amine group, it forms a branched structure by conjugating to amine groups of basic amino acid side chains (Fig. 2). In addition, the number of the conjugated NLSs in an artificial substrate is very high. To study the effect of these structural anomalies, the in vitro transport reaction of artificial transport substrates synthesized by conjugating different numbers of SV 40 virus large T antigen NLS to PE by the aid of two different cross-linkers has been characterized, and compared with the transport reaction of a natural nuclear protein.

The active transport of PE-NLS_{MBS}, PE-$NLS_{SMCC4\text{-}5}$ or Bsp Mase-NLS_2 without any exogenously added protein factor indicates that the nuclear transport is not absolutely dependent on soluble cytosolic proteins. The cytosol independent transport exhibits all the essential properties of an authentic in vivo transport process: it requires the presence of a functional NLS on the transport substrate, it is temperature and ATP dependent, it is inhibited by lectin WGA, and it is saturable in respect of a functional NLS. This transport is not due to damage of the nuclear envelope, because just those nuclei which accumulate these transport substrates prevent the access of an anti-DNA antibody to the nuclear DNA. Further, this transport is not due to inefficient extraction of the cytosolic proteins during the permeabilization step.

The nuclear transport of PE-$NLS_{SMCC7\text{-}8}$, in sharp contrast, was absolutely dependent on exogenous cytosolic proteins. The two steps of the nuclear transport, cytosol-dependent but ATP independent targeting to the nuclear envelope and cytosol and ATP-dependent translocation steps, have clearly been reproduced with this substrate (Fig. 8C and D).

Transport experiments performed with different substrate combinations (Fig. 8H-K) strongly suggest that distinct transport mechanisms operate for different substrates in digitonin permeabilized cells. The distinction may either be due to the total number of the conjugated NLSs or the location of NLSs within the protein. At low NLS/protein ratio (up to 4-5 NLSs/subunit) the transport efficiency of PE-NLS and Bsp Mase-NLS was proportional to the number of conjugated NLSs. Thus, it is unreasonable to suppose that the inhibition of nuclear transport of the highly conjugated substrate is simply due to the total number of NLSs. It is tempting to



speculate that in the cytoplasm a retention system operates, which binds and retains nuclear proteins having two or more NLSs at a proper distance or in a proper three-dimensional arrangement. The release of this retention requires cytosolic proteins. A growing number of proteins have been shown to have two independent NLSs (Garcia-Bustos et al., 1991; Takemoto et al., 1994). In some cases the two signals have been proposed to be functionally distinct (Hall et al., 1990). Recently it has been shown that the nuclear transport of certain proteins is precisely regulated. The glycocorticoid receptor, a nuclear protein with dual NLS, is cytoplasmic in the absence of glycocorticoid and nuclear after hormone binding (Pickard and Yamamoto, 1987). Similarly, the nuclear transport of NF-kB, a heterodimer of the p50 and p65 NLS containing subunits is under strict control. Binding of the inhibitory IkB subunit blocks the nuclear transport of the heterodimer, while a phosphorylation dependent dissociation of IkB releases the block of the transport. Although masking of the NLSs on the p50 and p65 subunits by IkB has been supposed (Zabel et al., 1993), other regulatory mechanisms cannot be ruled out. Regulated cytoplasmic retention has recently been proposed for the *Xenopus* MyoD protein (Rupp et al., 1994).

The substrate-dependent distinct transport mechanisms may explain the controversial role of Ran/TC4 in the translocation step of the nuclear transport. Ran/TC4 in its GTP-bound form was shown to be required for the translocation of HSA-NLS_{SMCC} in permeabilized rat liver cells (Shannon Moore and Blobel, 1993; Melchior et al., 1993), but a mutant Ran/TC4, defective in GTP-binding, which severely inhibited nuclear growth and DNA synthesis, did not impair significantly the nuclear transport of HSA-NLS_{MBS} in a *Xenopus* in vitro transport system or in digitonin permeabilized tissue culture cells (Kornbluth et al., 1994).

This work was supported by grants from the National Scientific Research Fund (OTKA 898 and T54O9). The authors thank Dr G. Tóth for his help in the peptide synthesis and Dr Gy. Hadlaczky and Dr J. Moitra for discussions and a critical reading of the manuscript.

REFERENCES

Adam, S. A., Lobl, T. J., Mitchell, M. A. and Gerace, L. (1989). Identification of specific binding proteins for a nuclear location sequence. *Nature* **337**, 276-279.

Adam, S. A., Sterne-Marr, R. and Gerace, L. (1990). Nuclear protein import in permeabilized mammalian cells requires cytoplasmic factors. *J. Cell Biol.* **111**, 807-816.

Adam, S. A. and Gerace, L. (1991). Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell* **66**, 837-847.

Adam, E. J. and Adam, S. A. (1994). Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. *J. Cell Biol.* **125**, 547-555.

Bonner, W. M. (1975). Protein migration into nuclei. I. Frog oocyte nuclei in vivo accumulate microinjected histones, allow entry to small proteins, and exclude large proteins. *J. Cell Biol.* **64**, 431-437.

Breeuwer, M. and Goldfarb, D. S. (1990). Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. *Cell* **60**, 999-1008.

Dingwall, C. (1991). Transport across the nuclear envelope: enigmas and explanations. *BioEssays* **13**, 213-218.

Dingwall, C. and Laskey, R. (1992). The nuclear membrane. *Science* **258**, 942-947.

Feldherr, C. M., Cohen, R. J. and Ogburn, J. A. (1983). Evidence for mediated protein uptake by amphibian oocyte nuclei. *J. Cell Biol.* **96**, 1486-1490.

Garcia-Bustos, J., Heitman, J. and Hall, M. N. (1991). Nuclear protein localization. *Biochim. Biophys. Acta* **1071**, 83-101.

Goldfarb, D. S., Gariepy, J., Schoolnik, G. and Kornberg, R. D. (1986). Synthetic peptides as nuclear localization signals. *Nature* **332**, 641-644.

Hadlaczky, Gy. and Praznovszky, T. (1988). Immunological similarities of eukaryotic chromosome components detected by human autoantibodies. *Genet. (Life Sci. Adv.)* **7**, 51-56.

Hall, M. N., Craik, C. and Hiraoka, Y. (1990). Homeodomain of yeast repressor a2 contains a nuclear localization signal. *Proc. Nat. Acad. Sci. USA* **87**, 6954-6958.

Hanover, J. A. (1992). The nuclear pore: at the crossroads. *FASEB J.* **6**, 2288-2295.

Hennekes, H., Peter, M., Weber, K. and Nigg, E. A. (1993). Phosphorylation on protein kinase C sites inhibits nuclear import of Lamin B_2. *J. Cell Biol.* **120**, 1293-1304.

Hurt, E. C. (1993). The nuclear pore complex. *FEBS Lett.* **325**, 76-80.

Kalderon, D., Richardson, W. D., Markham, A. T. and Smith, A. E. (1984). Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature* **311**, 33-38.

Kornbluth, S., Dasso, M. and Newport, J. (1994). Evidence for a dual role for TC4 protein in regulating nuclear structure ans cell cycle progression. *J. Cell Biol.* **125**, 705-719.

Lanford, R. E. and Butel, J. S. (1984). Construction and characterization of an SV 40 mutant defective in nuclear transport of T antigen. *Cell* **37**, 801-813.

Lanford, R. E., Kanda, P. and Kennedy, R. C. (1986). Induction of nuclear transport with a synthetic peptide homologous to the SV 40 T antigen transport signal. *Cell* **46**, 575-582.

Melchior, F., Paschal, B., Evans, J. and Gerrace, L. (1993). Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J. Cell Biol.* **123**, 1649-1659.

Newmeyer, D. D., Lucocq, J. M., Burglin, T. R. and De Robertis, E. M. (1986a). Assembly in vitro of nuclei active in nuclear protein transport: ATP is required for nucleoplasmin accumulation. *EMBO J.* **5**, 5021-510.

Newmeyer, D. D., Finlay, D. R. and Forbes, D. J. (1986b). In vitro transport of a fluorescent nuclear protein and exclusion of non-nuclear proteins. *J. Cell Biol.* **103**, 2091-2102.

Newmeyer, D. D. and Forbes, D. J. (1988). Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. *Cell* **52**, 641-653.

Newmeyer, D. D. and Forbes, D. J. (1990). An N-ethylmaleimide-sensitive cytosolic factor necessary for nuclear protein import: requirement in signal-mediated binding. *J. Cell Biol.* **110**, 547-557.

Newmeyer, D. D. (1993). The nuclear pore complex and the nucleocytoplasmic transport. *Curr. Opin. Cell Biol.* **5**, 395-407.

Newport, J. (1987). Nuclear reconstitution in vitro: stages of assembly around protein-free DNA. *Cell* **48**, 205-217.

Nigg, E. A., Baeuerle, P. A. and Lührmann, R. (1991). Nuclear import-export: in search of signals and mechanisms. *Cell* **66**, 15-22.

Osborn, M. A. and Silver, P. A. (1993). Nucleocytoplasmic transport in the yeast Saccharomyces cerevisiae. *Annu. Rev. Biochem.* **62**, 219-254.

Paine, P. L., Moore, L. C. and Horowitz, S. B. (1975). Nuclear envelope permeability. *Nature* **254**, 109-114.

Peters, R. (1986). Fluorescence microphotolysis to measure nucleocytoplasmic transport and intracellular mobility. *Biochim. Biophys. Acta* **864**, 305-359.

Pickard, D. and Yamamoto, K. (1987). Two signals mediate hormone-dependent nuclear localization of the glycocorticoid receptor. *EMBO J.* **6**, 3333-3340.

Pósfai, Gy., Kiss, A. and Venetianer, P. (1986). Overproduction of the Bacillus sphericus R modification methylase in E. coli and its purification to homogeneity. *Gene* **50**, 63-67.

Richardson, W. D., Mills, A. D., Dilworth, S. M. Laskey, R. A. and Dingwall, C. (1988). Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. *Cell* **52**, 655-664.

Roberts, B. L., Richardson, W. D. and Smith, A. E. (1987). The effect of protein context on the nuclear location signal function. *Cell* **50**, 465-475.

Rupp, R. A. W., Snider, L. and Weintraub, H. (1994). Xenopus embryos regulate the nuclear localization of XMyoD. *Genes Dev*. **8**, 1311-1323.

Shannon Moore, M. and Blobel, G. (1992). The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell* **69**, 939-950.

Shannon Moore, M. and Blobel, G. (1993). The GTP-binding protein

Ran/TC4 is required for protein import into the nucleus. *Nature* **365**, 661-663.

Shi, Y. and Thomas, J. O. (1992). The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Mol. Cell. Biol.* **12**, 2186-2192.

Silver, P. A. (1991). How proteins enter the nucleus. *Cell* **64**, 489-497.

Sterne-Marr, R. Blevitt, J. M. and Gerace, L. (1992). O-linked glycoproteins of the nuclear pore complex interact with a cytosolic factor required for the nuclear protein import. *J. Cell Biol.* **116**, 271-280.

Stewart, J. M. and Young, J. D. (1984). Solid phase synthesis of peptides. *Solid Phase Peptide Synthesis.* 2nd edn (ed. J. M. Stewart and J. D. Young), pp. 71-95. Pierce Chemical Company, New York.

Stochaj, U. and Silver, P. A. (1992). A conserved phosphoprotein that specifically binds nuclear localization sequences is involved in nuclear import. *J. Cell Biol.* **117**, 473-482.

Stochaj, U., Bossie, M. A., van Zee, K., Whalen, A. M. and Silver, P. A. (1993). Analysis of conserved binding proteins for nuclear localization sequences. *J. Cell Sci.* **104**, 89-95.

Takemoto, Y., Tashiro, S., Handa, H. and Ishii, S. (1994). Multiple nuclear localization signals of the B-myb gene product. *FEBS Lett.* **350**, 55-60.

Udvardy, A. (1993). Purification and characterization of a multiprotein component of the Drosophila 26S (1500 kDa) proteolytic complex. *J. Biol. Chem.* **268**, 9055-9062.

Yamasaki, L., Kanda, P. and Lanford, R. E. (1989). Identification of four nuclear transport signal-binding proteins that interact with diverse transport signals. *Mol. Cell. Biol.* **9**, 3028-3036.

Zabel, U., Henkel, T, dos Santos Silva, M. and Baeuerle, P. (1993). Nuclear uptake control of NF-kB by MAD-3 an IkB protein present in the nucleus. *EMBO. J.* **12**, 201-211.

(Received 25 November 1994 - Accepted 23 January 1995)