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Nuclear Import Can Be Separated into Distinct Steps In Vitro: Nuclear Pore Binding and Translocation

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Summary

Large nuclear proteins must possess a signal sequence to pass through the nuclear pores. Using an in vitro system, we have been able experimentally to dissect nuclear protein transport into two distinct steps: binding and translocation. In the absence of ATP, we observe a binding of nuclear proteins to the pore that is signal sequence-dependent. Translocation through the pore, on the other hand, strictly requires ATP. These steps, visualized in the fluorescence and electron microscopes, were observed both with a natural nuclear protein, nucleoplasmin, and a synthetic nuclear protein, composed of the signal sequence of SV40 T antigen coupled to HSA. When a mutant signal sequence was coupled to HSA, neither transport nor binding were observed, indicating that both result from the presence of a functional signal sequence. An inhibitor of transport, the lectin WGA, also arrested nuclear proteins in a bound state at the cytoplasmic face of the pore. Therefore, only the translocation step is sensitive to the inhibitor WGA, which is known to bind specifically to proteins of the nuclear pore.

Introduction

The nuclear pore forms a large channel connecting nuclear and cytoplasmic environments. Transport through the pore is fundamentally different from transport of proteins into mitochondria or endoplasmic reticulum in that it occurs through a large aqueous channel. In this respect, the nuclear pore resembles intercellular channels such as the gap junction, an aqueous channel 16–20 Å in diameter (Schwartzmann et al., 1981). The 90–100 Å channel of the nuclear pore apparently poses no barrier to small molecules and proteins, but the pore regulates the movement of large nuclear proteins and RNA molecules (Bonner, 1978; for reviews, see Franke, 1974; Maul, 1977; De Robertis, 1983; Dingwall and Laskey, 1986; Newport and Forbes, 1987). As in mitochondrial and ER transport, transport of large proteins into the nucleus requires the presence of a signal sequence.

Possession of a signal sequence by large nuclear proteins appears to allow the effective diameter of the pore to expand to allow their entry into the nucleus. Early indications that nuclear signal sequences exist came from studies of nucleoplasmin, a pentameric protein ~100 kd in size (Krohne and Franke, 1980; Dingwall et al., 1982; Dingwall et al., 1987; Bürglin and De Robertis, 1987). Nucleoplasmin and many other nuclear proteins can reenter the nucleus when microinjected into the cytoplasm (Gurdon, 1970; Bonner, 1975a, 1975b; De Robertis et al., 1978), arguing that the propensity of a protein to migrate into the nucleus is conferred by an uncleaved signal in the polypeptide chain. By complexing nucleoplasmin to gold particles up to 200 Å in diameter, Feldherr et al. (1984) observed that the nucleoplasmin signal sequence can confer upon gold particles the ability to be transported through the pores of Xenopus oocyte nuclei.

More recently, molecular genetic studies have shown that nuclear transport signals consist of short amino acid stretches. The precise signal sequences of a number of nuclear proteins are now known (Kalderon et al., 1984a, 1984b; Lanford and Butel, 1984; Hall et al., 1984; Silver et al., 1984; Davey et al., 1985; Moreland et al., 1985; Krippl et al., 1985; Lanford et al., 1986; Richardson et al., 1986; Richter et al., 1985; Wychowski et al., 1986; Bürglin and De Robertis, 1987). The best characterized signal, that of simian virus 40 (SV40) large T antigen (Lanford and Butel, 1984; Kalderon et al., 1984a, 1984b), consists of seven amino acids: pro-lys-lys$_{128}$-lys-arg-lys-val. A point mutation in this sequence (lys$_{128}$ to thr or asp) leads to a defect in nuclear transport of the T antigen. Goldfarb et al. (1986) and Lanford et al. (1986) showed that synthetic peptides containing the wild-type SV40 signal sequence, when covalently coupled to nonnuclear proteins and injected into living cells, cause those proteins to be imported into the nucleus. In contrast, proteins coupled to peptides containing point mutations at lys$_{128}$ are transport-defective. Such peptide-protein conjugates have been used by Goldfarb et al. (1986) to show that transport into the Xenopus oocyte nucleus displays saturable kinetics, i.e., coinjection of large amounts of free signal peptide inhibits the import of labeled signal-sequence-protein conjugates, arguing that transport is a receptor-mediated process.

Recently, in vitro nuclear transport systems have begun to allow the experimental manipulation, not just of the signal sequence, but of the machinery of nuclear protein import (Newport and Forbes, 1985; Newmeyer, 1986a, 1986b; Dreyer et al., 1986; Peters et al., 1986; Finlay et al., 1987). These systems, based on Xenopus egg extracts, take advantage of an unusual feature of the amphibian egg: storage in the unfertilized egg of all the materials, except DNA, needed to assemble thousands of nuclei (summarized by Laskey et al., 1979; Forbes et al., 1983). Such extracts can form nuclei from added sperm chromatin (Lohka and Masui, 1983, 1984; Dreyer et al., 1986) or from DNA alone (Newport and Forbes, 1985; Newmeyer et al., 1986a, 1986b; Blow and Laskey, 1986; Newport, 1987).

In a previous paper, we described an in vitro assay for nuclear import composed of an extract of Xenopus eggs, nuclei from any of several sources, and a fluorescently labeled nuclear protein, TRITC-nucleoplasmin (tetramethylrhodamine isothiocyanate; Newmeyer et al., 1986b). Isolated mammalian nuclei import nuclear proteins efficiently when placed in the assay mix, but exclude large

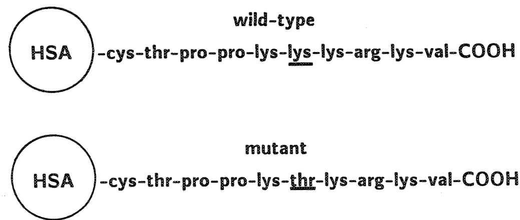

Figure 1. The Sequences of the Synthetic Peptides Containing Wild-Type and Mutant SV40 T Antigen Nuclear Targeting Signals Complexed to HSA for This Study

nonnuclear proteins. The import of nucleoplasmin in this system displays the characteristics predicted for an active transport system: transport requires a signal domain, is completely ATP-dependent, is temperature-dependent, and requires an intact nuclear envelope. With this system, we found that the lectin wheat germ agglutinin (WGA) inhibits nucleoplasmin transport by binding to sugar moieties in the nuclear pore (Finlay et al., 1987) and concluded that pore complex glycoproteins play a role in the transport mechanism. In fact, at least eight different WGA-binding glycoproteins have recently been identified as components of the nuclear pore (see Discussion).

Although little of the molecular mechanism of nuclear protein import, i.e., signal sequence recognition, channel opening, or the nature of the proteins involved, is as yet understood, the observations that nucleoplasmin transport is ATP-dependent and inhibited by WGA provide unique tools for investigating the transport process in detail. In this paper, we have made use of these observations in order to examine the interactions of nuclear transport signals with the nuclear pore. Our results show that nuclear transport can be experimentally separated into two distinct steps: binding and translocation. The first step, binding to the pore, is due to specific recognition of the transport signal, does not require ATP, and is not inhibited by WGA. The second step, translocation through the pore, is ATP-dependent and WGA-sensitive.

Results

Specific Recognition of the SV40 T Antigen Nuclear Localization Signal in an In Vitro Transport System

To study the recognition of nuclear signal sequences in our in vitro transport system, peptides were synthesized that correspond to the wild-type or mutant transport signals of SV40 T antigen (Figure 1). The mutant signal contains a single amino-acid substitution (lys_{128} to thr) known to lead to a transport defect for T antigen in vivo (Kalderon et al., 1984a, 1984b). These peptides were coupled to human serum albumin (HSA) using the heterobifunctional cross-linker MBS. For detection in the fluorescence microscope, peptide-HSA conjugates were tagged with TRITC. The average number of peptides per HSA molecule was estimated by gel electrophoresis to be ~20 and ~18 peptides per molecule of HSA for the wild-type and mutant signal sequences, respectively. Conjugates of wild-type

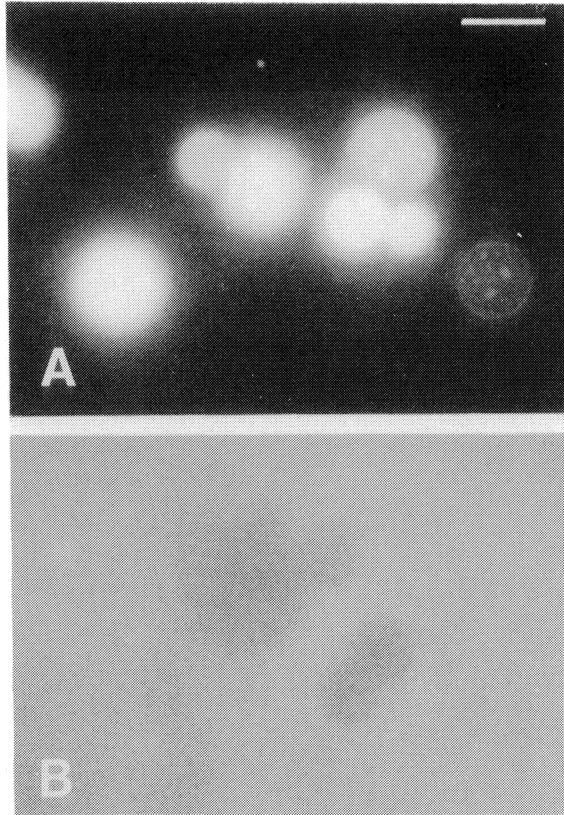

Figure 2. Transport of TRITC-wt-HSA Conjugate into Rat Liver Nuclei and Exclusion of TRITC-Mutant-HSA Conjugate from Rat Liver Nuclei

Rat liver nuclei were added to an extract of Xenopus eggs and incubated for 30 min before addition of TRITC-labeled conjugate. After a further 30 min incubation, aliquots of each transport assay mix were fixed and photographed using the fluorescence microscope. (A) TRITC-wt-HSA fluorescence. (B) TRITC-Mutant-HSA. Bar indicates 10 μm.

peptide and mutant signal sequence peptide are referred to below as "wt-HSA" and "mutant-HSA."

To test the ability of these peptides to act as nuclear targeting signals in vitro, the TRITC-labeled peptide-HSA conjugates were added to our nuclear import assay, which consists of an extract of Xenopus eggs, rat liver nuclei, and an ATP-regenerating system (Newmeyer et al., 1986b). We found that TRITC-wt-HSA conjugate was transported efficiently into rat liver nuclei (45-fold accumulation after 30 min; Figure 2A), whereas the mutant conjugate was excluded from nuclei (<1-fold accumulation; Figure 2B). We concluded that the SV40 T antigen nuclear transport signal is recognized specifically in this in vitro system, as it is in vivo.

To demonstrate transport in vitro at the level of the individual nuclear pore, we used the electron microscopic method of Feldherr et al. (1984). wt-HSA and mutant-HSA were coupled to 10 nm colloidal gold particles, then added to the normal transport assay mix containing rat liver nuclei, and incubated for 30 min. Electron microscopy of sections of the sample revealed that wt-HSA-coated gold

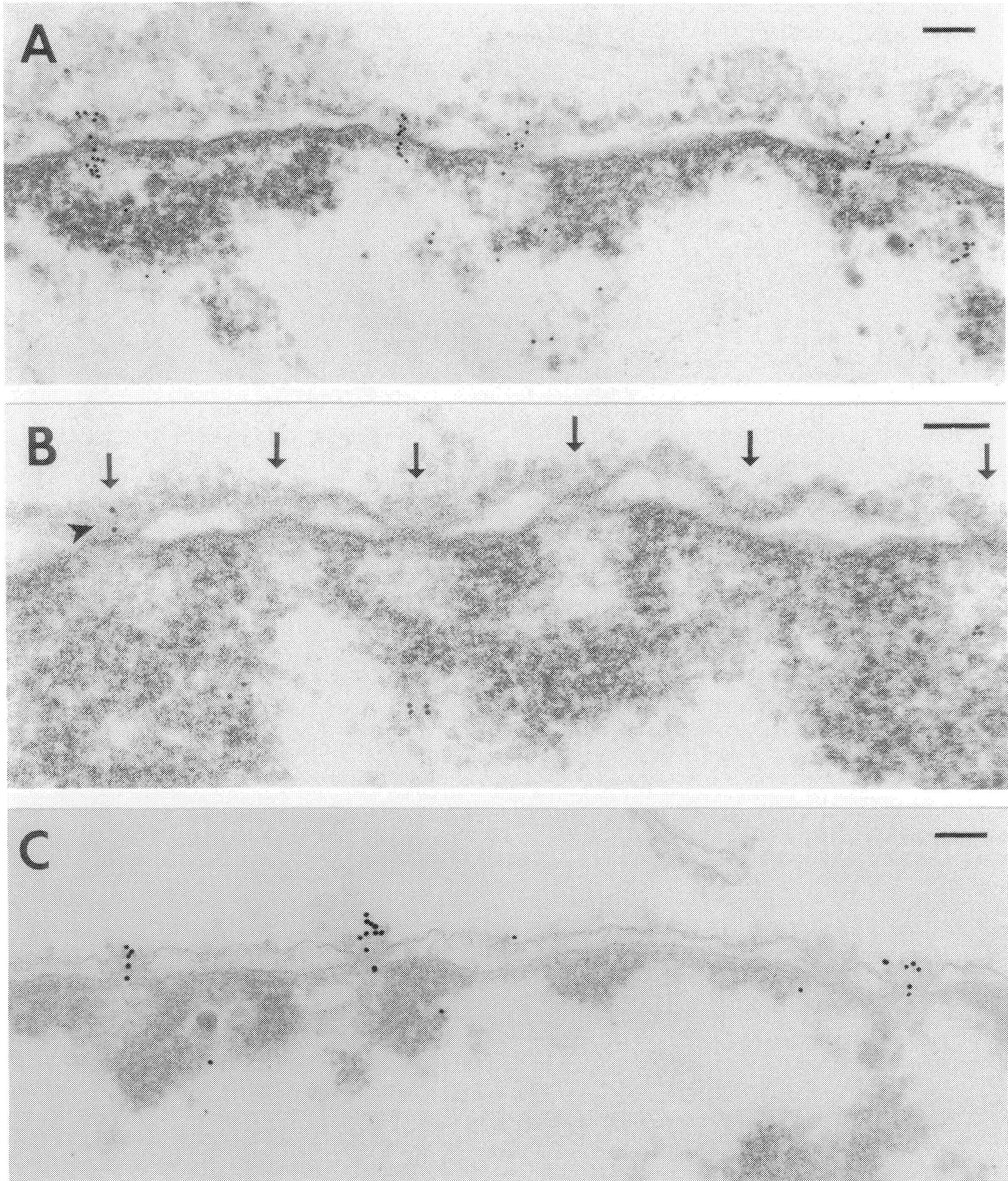

Figure 3. wt-HSA-Coated and Nucleoplasmin-Coated Gold Particles Are Translocated through Nuclear Pores While Mutant-HSA-Coated Gold Particles Lack Strong Interaction with the Pores

Colloidal gold particles coated with wt-HSA (A), mutant-HSA (B), or nucleoplasmin (C) were added to nuclei in the standard transport assay. Thirty minutes later, the samples were diluted, fixed with glutaraldehyde, the nuclei concentrated, and prepared for electron microscopy as described in Experimental Procedures. Nuclear envelopes typical of each experimental condition are shown. Each panel is oriented so that the interior of the nucleus containing the chromatin is at the bottom of photograph. It should be noted that little if any binding to other organelles or membranes was observed. Bars indicate 100 nm.

particles were translocated efficiently through the central channels of the nuclear pores (that is, the narrow constrictions at the "waists" of the pores) and accumulated in the nuclear interior. Typically, as can be seen in Figure 3A, the majority of pores showed multiple gold particles in transit, i.e., in the channel or having passed through the channel.

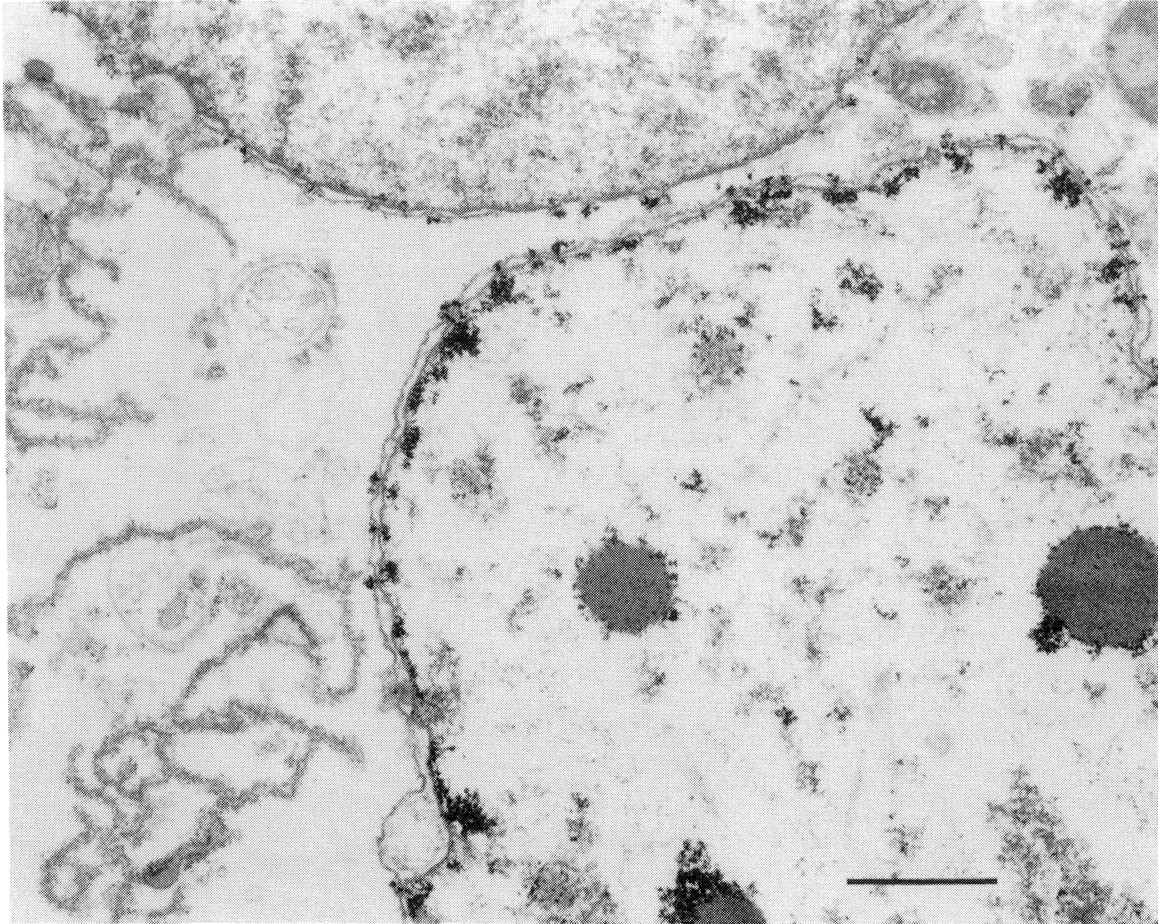

Figure 4. High Amounts of wt-HSA-Coated Gold Particles Can Be Translocated Efficiently through the Nuclear Pores
The experiment was as in Figure 3A, but three to four times the concentration of wt-HSA gold was added. Portions of two nuclei are shown. Little binding to other organelles or membranes is seen. The dense spherical bodies in the nucleus are nucleoli. Bar indicates 1000 nm.

So efficient was transport that, even in the bright-field microscope, the reddish-brown color of wt-HSA-coated colloidal gold became visible in many nuclei as the assay proceeded. When experiments were performed with nucleoplasmin-coated gold, results similar to those seen with wt-HSA were observed (Figure 3C). The nucleoplasmin-coated gold particles were translocated through the nuclear pores and all pores observed were active in transport.

In contrast, when gold particles coated with mutant-HSA were used, an occasional pore was seen with one or two associated gold particles, but the majority of pores were completely devoid of gold particles (Figure 3B). Thus, in the in vitro system, wt-HSA is transported through the nuclear pores and accumulated within the nucleus while mutant-HSA is transported extremely poorly.

When we added 3- to 4-fold higher amounts of wt-HSA-coated gold to the assay, we observed at least a 3-to 4-fold higher number of gold particles being translocated through the pores (Figure 4). Transport under these conditions was very efficient. Large numbers of gold particles could be seen on the nucleoplasmic side of the pore. We conclude that the experiments shown in Figure 3 employed subsaturating levels of transport substrates. Once in the nucleus wt-HSA-coated particles had some affinity for the nucleoli (the dense spherical bodies), an affinity visible in fluorescence experiments (see Figure 2A). However, this nucleolar association appeared to be a minor fraction of the total wt-HSA protein in the nuclei in both types of experiments. Figure 4 also demonstrates that, although the transport assay extracts contain large amounts of Xenopus organelles and membrane vesicles, none of these show significant association with wt-HSA gold particles, indicating that the binding of this signal sequence is specific to the pore. We also found that when the large concentrations of mutant-HSA-coated gold particles were used, a number of particles became associated with the pores (data not shown), although to a much lower extent than the wt-HSA under similar conditions (Figure 4). This last result is in agreement with that of Goldfarb et al. (1986), who found that the point mutation at lys_{128} did not abolish transport but reduced the final level of nuclear accumula-

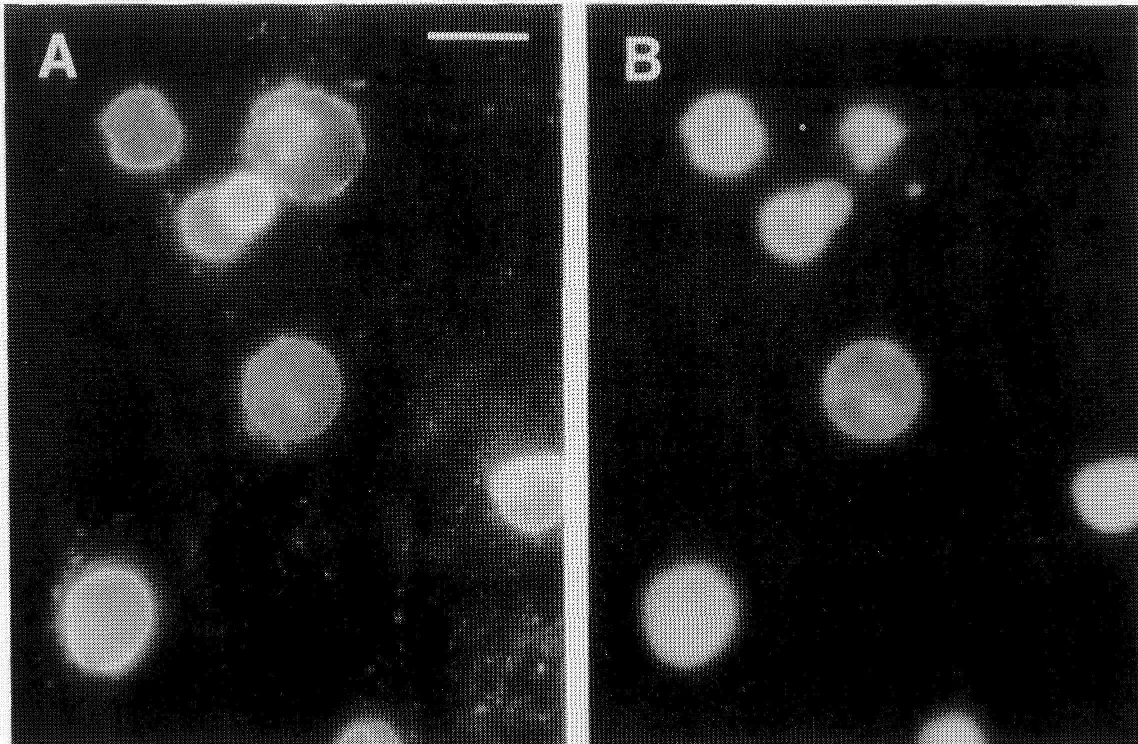

Figure 5. Binding of Fluorescent wt-HSA to the Nuclear Envelope Occurs in the Absence of ATP

Nuclei were added to egg extract and incubated for 30 min. At this time, apyrase was added to 100 U/ml and incubated an additional 30 min prior to the addition of TRITC-wt-HSA. The nuclei were observed in the fluorescence microscope 30 min after TRITC-wt-HSA was added. (A) TRITC-wt-HSA showing fluorescent staining of the nuclear periphery. (B) Nuclear DNA stained by the fluorescent DNA dye, Hoechst 33258. Bar indicates 10 μm.

tion 6- to 7-fold and suggests that the mutant sequence functions as a very weak transport signal both in vivo and in vitro.

Nuclear Protein Import: Separable Binding and Translocation Steps

Nuclear transport has been shown to be ATP-dependent (Newmeyer, et al. 1986a, 1986b). Specifically, previous studies found that the nuclear accumulation of nucleoplasmin is completely inhibited in the absence of ATP. We found that the removal of ATP from the transport extract with the enzyme apyrase also blocked accumulation of TRITC-wt-HSA (Figure 5). Interestingly, however, under conditions of ATP-depletion, TRITC-wt-HSA strongly labeled the nuclear envelope, as seen in Figure 5A. TRITC-wt-HSA containing lower numbers of wild-type signal sequence peptide per HSA molecule showed proportionately less envelope binding (data not shown). When fluorescently labeled mutant-HSA was tested under similar ATP-depleted conditions, it was neither transported nor found to bind significantly to the nuclear envelope (electron microscopy presented below). The binding of fluorescent wt-HSA to the nuclear envelope was completely inhibited by addition of a large excess of unlabeled wt-HSA, but not by excess mutant HSA. Taken together, these results indicate that the observed binding to the nuclear envelope involves specific recognition of a functional transport signal.

Using electron microscopy, we asked whether wt-HSA-coated gold particles were binding to the nuclear pore in the absence of ATP or whether the binding was nonspecifically localized over the entire envelope. We observed that ATP depletion with the enzyme apyrase blocked translocation of wt-HSA-coated gold particles through the nuclear pores (Figure 6A). No gold particles were found within nuclei and none in the central channels of the nuclear pores, i.e., no gold particles were found to have crossed the "waists" of the pores. Although they were not translocated, gold particles were seen to be bound to the cytoplasmic faces of the pores, associated with amorphous electron-dense material that may correspond to the annular granules (see Unwin and Milligan, 1982). In both cross section (Figure 6A) and en face views (data not shown), as many as 20 gold particles were found associated with an individual pore, indicating that there are multiple binding sites for nuclear signal sequences on each pore. Identical results were obtained when ATP was depleted with a different enzyme, hexokinase (data not shown). We conclude that ATP is required for translocation through the pore but not for binding to sites on the cytoplasmic face of the pore.

When nucleoplasmin gold was used instead of wt-HSA gold under conditions of ATP depletion, again binding of multiple gold particles to the pores was seen but not translocation or accumulation (see Table 1). Thus, at the electron microscope level, the nucleoplasmin and wt-HSA

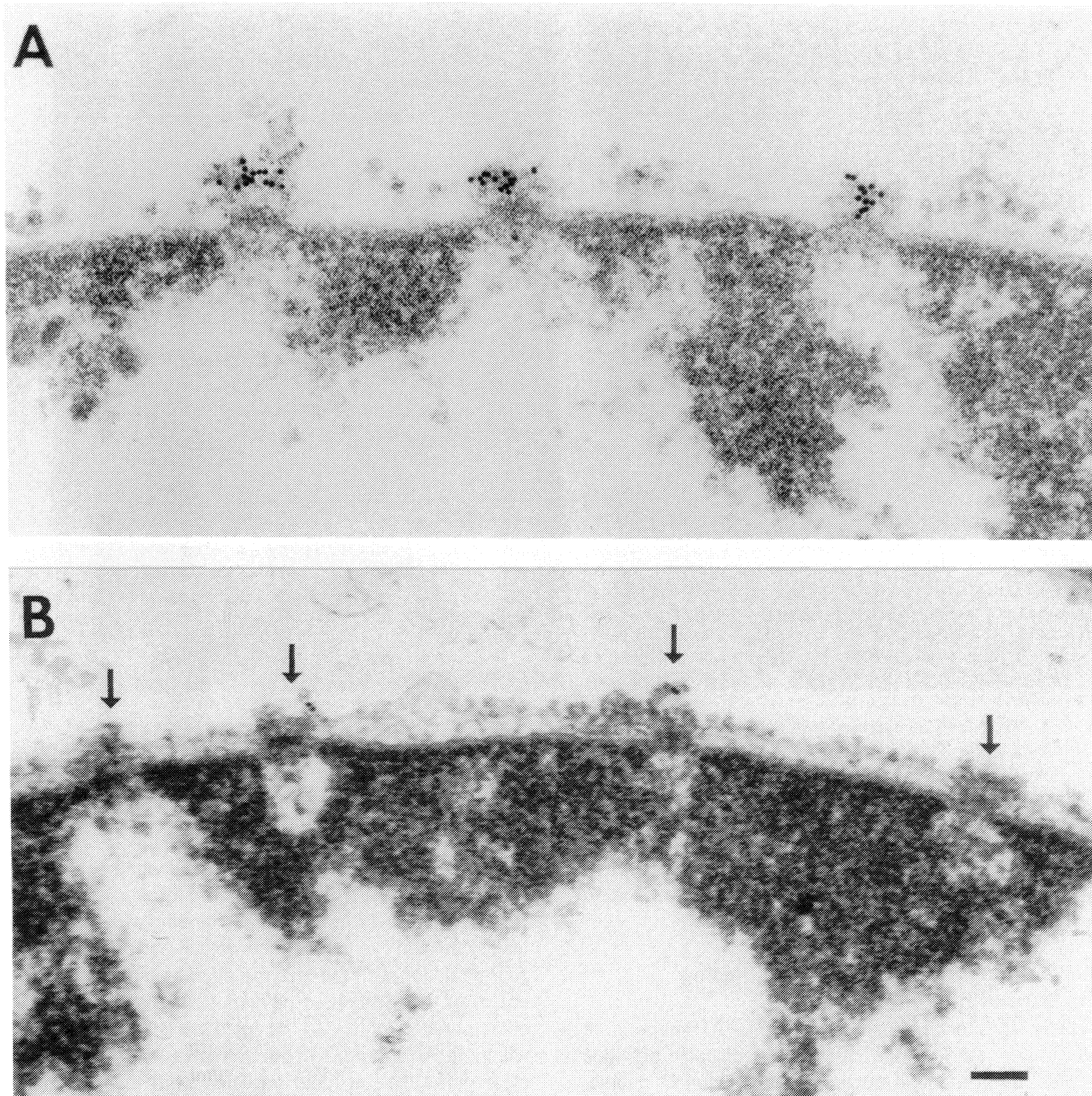

Figure 6. Binding of wt-HSA-Coated Gold Particles, but Not Mutant-HSA-Coated Gold Particles, to the Pore in the Absence of ATP
Nuclei were added to a standard transport assay. ATP was then depleted by the addition of apyrase for 30 min. (A) wt-HSA-coated colloidal gold particles were added and the nuclei were fixed and prepared for electron microscopy 30 min later. A section of a typical nucleus containing three visible pores is shown, each binding multiple gold particles. (B) Mutant-HSA-coated gold was added and processed as above. A section of a typical nucleus is shown with the four nuclear pores designated with arrows. Each panel is oriented so that the nuclear interior is located in the lower half of the panel. Bar indicates 100 nm.

results are identical. The electron microscopic technique appears to be much more sensitive in detecting low levels of binding or accumulation, since fluorescent nucleoplasmin did not show obvious binding to the nuclear envelope. This would be expected for a technique that is capable of showing single gold particles associated with the pore.

When mutant-HSA-coated gold particles were added to nuclei in the transport assay after ATP depletion, binding to a pore was observed only rarely. As in conditions where ATP was present, the majority of pores showed no associated gold particles (Figure 6B). Translocation and accumulation of mutant-HSA-coated particles were also not observed. Thus, the binding to the pore that we observe with wt-HSA-coated gold and nucleoplasmin-coated gold when ATP is depleted requires a correct signal sequence.

A Nuclear Transport Inhibitor, WGA, Blocks the Translocation Step but Not the Binding Step

Although relatively little is known about the biochemical makeup of the pore, we previously demonstrated that the

Table 1. Summary of Fluorescence and Electron Microscopic Experiments

	Fluorescence[a]			Electron Microscopy[b]	
	Exclusion	Binding	Transport	Pore Binding	Transport
SV40-Signal-HSA					
wt-HSA	−	+ + +	+ + +	+ + +	+ + +
mut-HSA	+	−	−	±	±
wt-HSA, apyrase ±[c]	+	+ + +	−	+ + +	−
wt-HSA, hexokinase ±[c]	+	+ + +	−	+ + +	−
mut-HSA, apyrase	+	−	−	±	−
wt-HSA + WGA	+	+ + +	−	+ + +	±
mut-HSA + WGA	+	−	−	±	−
wt-HSA + WGA + sugar	−	+ + +	+ +	+ + +	+ +
wt-HSA (high amounts)	n.d.	n.d.	n.d.	+ + + +	+ + + +
mut-HSA (high amounts)	n.d.	n.d.	n.d.	+ +	+
Nucleoplasmin					
NP	−		+ +	+	+ +
NP, apyrase ±[c]	−[c]		−	+ +	−
NP + WGA	+		−	+ +	±
NP + WGA + sugar	−		+ +	+ +	+

Symbols used to designate maximum observed level of transport of fluorescent substrates; +, ~3-fold accumulation over background; + +, ~15-fold accumulation; + + +, ~45-fold accumulation. Fluorescence experiments were performed numerous times and quantitated by densitometry of photographic negatives.

Symbols used to designate binding; ±, binding of 1–2 particles to an occasional pore; +, binding of 1–2 particles to the majority of pores; + +, binding of 2–5 particles per pore; + + +, binding of up to ~10 particles per pore; + + + +, binding of ~10–30 particles per pore.

Symbols used to designate transport: −, no gold particles passing through the lumen of the pore or in the nuclear interior (see Figure 6A for an example); ±, an occasional gold particle in the center of the pore or inside the nucleus (see Figure 3B); + +, 1–5 gold particles in transit through the lumen of the majority of pores (see Figure 3C); + + +, 5–15 gold particles in transit through the pore (see Figure 3A); + + + +, 20–100 gold particles in transit through the pore (see Figure 4). Electron microscopic experiments were performed multiple times, and each designation is the result of inspection of the pores of numerous nuclei.

[a] Performed with TRITC-labeled substrates.

[b] Performed with gold-labeled substrates.

[c] Equilibration instead of exclusion was seen. However, the nuclei continued to exclude FITC-labeled non-nuclear proteins.

n.d., not determined.

pore contains components that interact with the lectin WGA and that this interaction completely blocks transport (Finlay et al., 1987). Several groups have identified pore proteins containing N-acetylglucosamine residues (see Discussion). Theoretically, WGA might act to inhibit transport either by blocking signal sequence recognition or translocation through the pore. To distinguish between these mechanisms of inhibition, we asked whether binding of signal sequence-HSA to the pore could occur when transport was blocked with WGA. TRITC-wt-HSA (0.01 mg/ml) was added to nuclei simultaneously with a concentration of FITC-WGA (fluorescein isothiocyanate; 0.2 mg/ml) sufficient to block transport of the signal sequence-containing conjugate. We observed strong binding of both wt-HSA and WGA (Figure 7, A through D). Close inspection in the fluorescence microscope revealed that TRITC-wt-HSA decorated the nuclear envelope with a punctate pattern (Figure 7A) similar to the pore binding previously seen with FITC-WGA (Finlay et al., 1987). Examination of these samples, which were labeled simultaneously with TRITC-wt-HSA and FITC-WGA, indicated that the nuclear binding sites for these molecules were essentially colocalized (Figure 7, A through D).

Although both WGA (Finlay et al., 1987) and wt-HSA bound to the nuclear pore, they did not compete with each other for binding. Unlabeled WGA at high concentrations (4.0 mg/ml) competitively inhibited the binding of FITC-WGA (0.1 mg/ml), but had no effect on the nuclear envelope binding of TRITC-wt-HSA (0.01 mg/ml; data not shown; see electron microscopic experiment below). Conversely, unlabeled wt-HSA at 2.0 mg/ml inhibited binding of TRITC-wt-HSA (0.01 mg/ml) but not FITC-WGA (0.05 mg/ml; data not shown). Thus, at concentrations of WGA much greater than that required to block translocation, fluorescently labeled signal sequence binding was not affected.

Electron microscopic experiments were performed using wt-HSA-coated gold particles. When nuclei were preincubated with WGA before addition of wt-HSA-coated gold, translocation across the pore was markedly inhibited (Figure 8). Prior addition of WGA did not, however, block the binding of wt-HSA gold to pores (Figure 8), nor did it block the binding of nucleoplasmin-coated gold (data not shown). In order to insure the blockage of all WGA sites, ten times the concentration of WGA required to block nuclear accumulation (as observed in the fluorescence microscope) was used in the above electron microscopic experiments. The electron microscopy demonstrates that WGA blocks translocation but not binding of the wt-HSA, and further, that the molecular sites in the

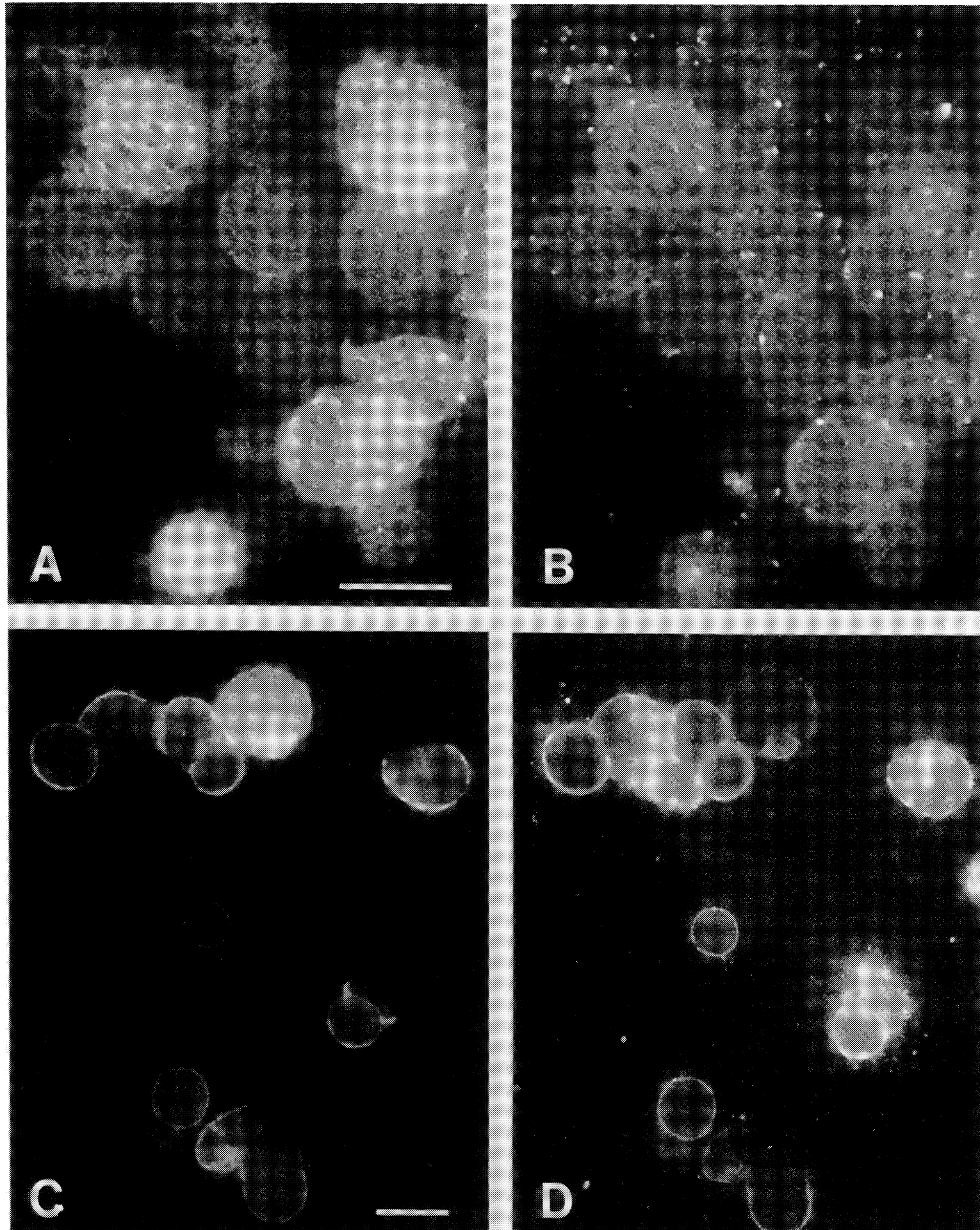

Figure 7. Binding of TRITC-wt-HSA to the Nuclear Envelope in the Presence of FITC-WGA

Nuclei incubated 30 min in the standard transport assay mix were treated with 0.2 mg/ml FITC-WGA for 5 min before addition of TRITC-wt-HSA. Thirty minutes later, the nuclei were examined for the location of the two fluorescently labeled substrates. The binding sites of TRITC-wt-HSA (A and C) were found to colocalize with the nuclear binding sites for FITC-WGA (B and D). (A) and (B): Plane of focus at the surface of the nuclei, showing a punctate pattern. (C) and (D): Plane of focus through the equator of the nuclei, showing a peripheral staining pattern. (A) and (C): TRITC-wt-HSA fluorescence. (B) and (D): FITC-WGA fluorescence. Note that this concentration of WGA is sufficient to block accumulation of TRITC-wt-HSA (C). Bars indicate 10 μm.

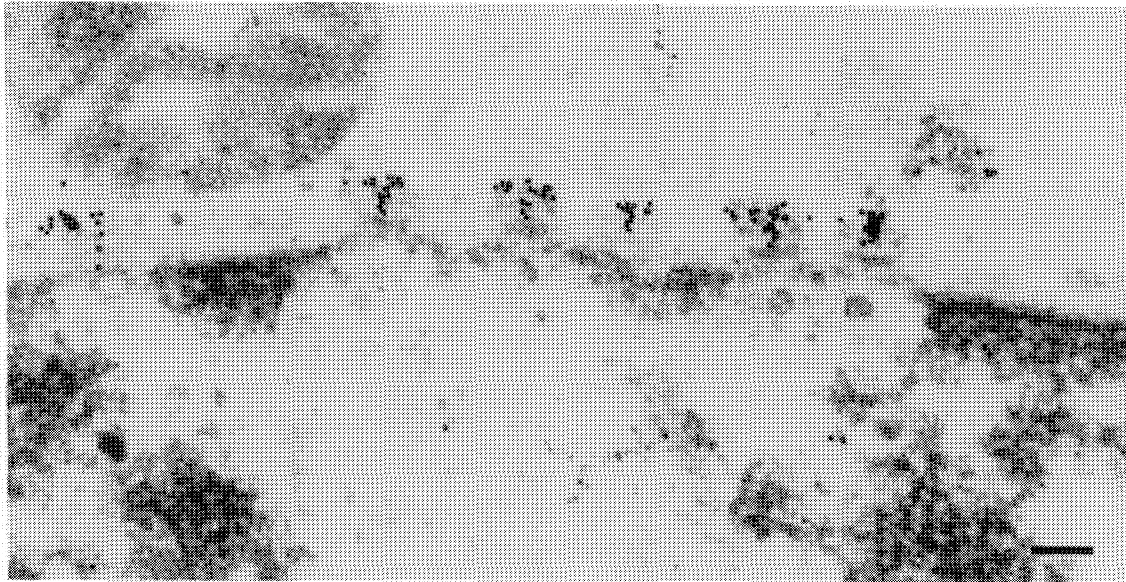

Figure 8. wt-HSA-Coated Gold Particles Bind to the Pore When Transport Is Blocked by the Presence of the Transport Inhibitor WGA
Nuclei were incubated in the transport assay for 30 min. At this time, WGA (1 mg/ml) was added to the nuclei and followed 5 min later by addition of wt-HSA-coated gold particles. Thirty minutes after addition of the gold, the nuclei were fixed and prepared for electron microscopy as described in Experimental Procedures. A section of a typical nucleus is shown with ~6 pores visible. The micrograph is oriented so that the nuclear interior lies in the lower half of the figure and the external medium in the upper half. The bar indicates 100 nm.

pore to which WGA binds to cause the block in translocation are distinct from the signal recognition sites. The above results and additional controls are summarized in Table 1.

Discussion

Fidelity of the In Vitro System

Using an in vitro system for nuclear transport, one can manipulate the events of transport in a way not possible in vivo. With such a system, we have divided the process of transport into two distinct events that are recognizable at the level of the individual pore. For the results of such experiments to be extrapolated to an in vivo context, it was first necessary to confirm that all of the observations that have been made in vivo with the transport substrates we use are retained in our in vitro experiments. For this purpose, we have tested in vitro each of the in vivo findings that use the SV40 T antigen signal sequence complexed to nonnuclear proteins. We can find no differences. HSA complexed to a synthetically produced wild-type SV40 signal sequence is transported into nuclei extremely efficiently. When complexed to gold, we estimate that the wt-HSA conjugate allows at least 100 gold particles to enter the nucleus through each pore in 30 min (see Figure 4). The conjugate of HSA with the wild-type signal sequence is, in fact, accumulated to a greater extent than the Xenopus nuclear protein, nucleoplasmin, a natural transport substrate (45-fold and 17-fold, respectively, as measured by the fluorescence assay). On the other hand, HSA conjugated to a mutant SV40 T antigen signal sequence that has been used as a paradigm of a signal sequence mutation in vivo (lys_{128} to thr_{128}; Kalderon et al., 1984a, 1984b; Goldfarb et al., 1986), is severely deficient in transport in vitro. Thus, we have established that in vitro these substrates mirror their behavior in vivo.

Recognition of the Signal Sequence Is Independent of and Precedes Translocation

Theoretically, nuclear transport could involve a cascade of events, each separate but required for triggering the next event. It is apparent from the study of larger nuclear proteins, dextrans, and the elegant in vivo work of Feldherr using nuclear proteins complexed to gold that one of these events must be an expansion of or increase in the effective pore channel (see Dingwall and Laskey, 1986; Newport and Forbes, 1987, for reviews; Feldherr et al., 1984). This event would be hypothesized to be preceded by recognition of a nuclear signal sequence and followed by translocation of the nuclear protein through the diaphragm of the pore. The steps of transport could thus be ordered: recognition of the signal sequence, pore expansion, and translocation of the nuclear protein through the expanded pore, either actively or passively. The translocation step itself might occur either by passive diffusion through the newly expanded channel or by some sort of motive system of the type found in actomyosin-dependent movement or kinesin-mediated movement along microtubules (Vale et al., 1985). At the experimental level, although one might be able to infer that a recognition event precedes and is separable from translocation by in essence taking kinetic "snapshots" of transport in vivo (see

for example Feldherr et al., 1984), it is only by blocking transport at intermediate steps that they can be proven to exist as independent events.

We find that by removing ATP from our in vitro assay we can indeed arrest transport after a signal-sequence recognition step. Specifically, both fluorescently labeled wt-HSA and wt-HSA complexed to gold particles bind tightly to nuclear pores but are not translocated through them when ATP is removed. Similarly, nucleoplasmin complexed to gold is seen to bind to the pore when ATP is depleted. We think that the stronger nuclear pore binding of TRITC-wt-HSA and wt-HSA gold is due to the presence of ~20 signal sequences per HSA molecule. The nucleoplasmin pentamer with five signal sequences (Bürglin et al., 1987; Dingwall et al., 1987; Bürglin and De Robertis, 1987; T. Bürglin, personal communication) binds, but does so less tightly. Consistent with this interpretation, we have in other studies tested directly the effect of changing the number of signal sequences in wt-HSA conjugates (Newmeyer and Forbes, in preparation). We find that wt-HSA with fewer signal sequences (~13) per molecule is accumulated much less in 30 min than a conjugate with a higher coupling ratio (~20) and shows only weak nuclear envelope binding under ATP-depleted conditions. A conjugate with approximately three signal sequences per HSA shows even less accumulation and very little binding. Both Dingwall et al. (1982) and Lanford et al. (1986) have found in vivo that the accumulation rate of nuclear proteins increases with increasing numbers of signal sequences per protein. From our in vitro results, we think that the number of signal sequences not only influences the rate of accumulation, but also affects the strength of nuclear pore binding in the absence of translocation. The stronger binding that we see with increasing signal sequence to protein ratios may result from either a multivalent interaction with the pore or a higher probability of rebinding after dissociation of the peptide-HSA conjugate.

We find that mutant-HSA gold in the presence of ATP is transported extremely poorly compared with wt-HSA gold (0.2 particles/pore versus 5–15 particles/pore, respectively). Under ATP-depleted conditions, mutant-HSA-coated gold binds to a small extent and is not transported. This is consistent with the interpretation that the mutant signal sequence binds to the pore, but with much lower affinity than the wild-type signal. The binding of TRITC-wt-HSA can be competed away by excess wt-HSA but not by excess mutant-HSA, again indicating that the mutant signal binds much less tightly to the nuclear pore than the wild-type signal. Our results agree qualitatively with the in vivo finding that this point mutation does not abolish transport in vivo, but reduces the rate of transport and the final extent of accumulation 6- to 7-fold (Goldfarb et al., 1986). We conclude then that binding of HSA-wt gold to the pore reflects a specific recognition of the wild-type transport signal.

Taken together, these results provide evidence for a recognition site in the nuclear pore that binds the transport signals of nucleoplasmin and SV40 T antigen. This binding step alone could account for the selectivity and saturability of nuclear import. ATP is clearly not required for the binding step. The binding sites observed with wt-HSA gold are localized in the area of the annular granules, with up to 20 gold particles being seen per pore. If we assume that the eight annular granules present on the cytoplasmic side of the pore are the site of signal sequence recognition, there must be a minimum of two to three binding sites associated with each annular granule.

ATP Is Required for an Event in Translocation through the Pore

The nuclear pore is a large structure 1200 Å in diameter (Unwin and Milligan, 1982). It has been determined from dextran and protein studies that the channel of the pore that is available for diffusion is only 90–110 Å in diameter (Paine and Horowitz, 1980; Peters, 1986; Peters et al., 1986). When a protein containing a signal sequence approaches the pore, the channel is capable of expanding its effective diameter to at least 200 Å (Feldherr et al., 1984). We find that, in the presence of ATP, signal sequence-coated gold particles can move through the channel of the pore. In the absence of ATP, however, the particles remain arrested in a bound state outside the channel. ATP could be required for the release of the particles from a signal sequence receptor, for the act of pore expansion, or for movement through the expanded pore. Equally, ATP may be needed for more than one of these steps. ATPases have been identified as present in the nuclear envelope (Yasuzumi and Tsubo, 1966; Scheer and Franke, 1969; Schröder et al., 1986; Berrios and Fisher, 1986), but have yet to be localized to the pore and thus may or may not be those involved in the ATP requirement we see for the translocation event.

The Transport Inhibitor WGA Acts at a Step Subsequent to the Binding Step

The transport and accumulation of nuclear proteins is inhibited by the lectin WGA, which binds in multiple copies to the nuclear pore (>15 copies/pore; Finlay et al., 1987). A family of approximately eight glycoproteins in the nuclear pore that contain N-acetylglucosamine residues and are thus capable of binding WGA have been recently identified (Holt and Hart, 1986; Davis and Blobel, 1986; Finlay et al., 1987; Schindler et al., 1987; Snow et al., 1987; Holt et al., 1987; Hanover et al., 1987; Park et al., 1987; Forbes et al., in preparation). One or all of these may be the target of WGA inhibition in the native pore. As an inhibitor of transport, WGA could theoretically act by blocking the first step in transport, i.e., signal sequence recognition. This clearly is not the primary mechanism of WGA inhibition: we find that in the presence of a vast excess of WGA, the signal sequence-HSA conjugate, either fluorescently labeled or gold-complexed, binds strongly to the nuclear pore. We conclude that WGA must bind to sites of translocation inhibition in the pore that are physically separate from the signal sequence receptor. Since WGA is 40 Å × 40 Å × 70 Å in diameter (Wright, 1977), the protein(s) with which WGA interacts to inhibit translocation would be predicted to be at least 20–35 Å distant from the protein involved in the initial binding of a nuclear-targeted protein.

Since concentrations of WGA that inhibit translocation do not inhibit binding to the pore, we conclude that WGA must act primarily to inhibit transport at a step beyond signal sequence recognition. In previous experiments, we found that WGA does not simply occlude the pore when it binds, thus preventing transport in that manner. In the presence of WGA, fluorescent 20 kd dextrans diffuse into the nucleus at a rate no different from that in the absence of WGA (Finlay et al., 1987; Finlay and Forbes, unpublished data). (In vivo, fluorescently labeled 20 kd dextrans can diffuse through the pores of rat hepatocyte nuclei while 40 kd dextrans are unable to enter the pore channel [Lang et al., 1986].) Instead, the data support a model where WGA, by binding to pore proteins, blocks either a pore-expansion step or, if this step is followed by an active translocation step, the movement of nuclear proteins through the pore channel.

In summary, the data presented indicate that selective transport through nuclear pores involves two experimentally separable events: binding and translocation. Binding involves specific recognition of the transport signal and is ATP-independent. In contrast, translocation through the pore is ATP-dependent and sensitive to the transport inhibitor, WGA. Since the fidelity of transport observed in vivo is retained in the in vitro system, it should be possible to dissect further the events of nuclear transport.

Experimental Procedures

Fluorescence Assay for Nuclear Transport

Extracts were prepared from Xenopus eggs according to methods previously published (Newmeyer et al., 1986b; Newport, 1987). Rat liver nuclei were isolated as described previously (Newmeyer et al., 1986b; Newport and Spann, 1987) using 250 mM sucrose, 80 mM KCl, 15 mM NaCl, 15 mM PIPES (pH 7.2), 1 mM DTT, 5 mM EDTA, 0.5 mM spermidine, 0.2 mM spermine, 1 μg/ml aprotinin, l μg/ml leupeptin, and 1 mM PMSF. It should be noted that, when incubated in egg extracts, many rat liver nuclei incorporate Xenopus nuclear envelope materials, often at a specific point in the rat envelope, so that the nucleus becomes double-lobed. The DNA usually remains in the original lobe of nuclear envelope, which is contiguous with an added lobe of envelope of Xenopus origin (Newmeyer et al., 1986b).

Nucleoplasmin and HSA-peptide conjugates were labeled with TRITC as previously described (Newmeyer et al., 1986b). The fluorescently labeled proteins were added (final concentration ~0.01 mg/ml) to egg extracts containing rat liver nuclei (~3000/μl) that had been allowed to incubate and heal in the extract for 30 min. Transport assays, ATP-depletion, and WGA treatment were performed as previously described (Newmeyer et al., 1986b; Finlay et al., 1987) except that the concentration of WGA was increased to 1.0 mg/ml, unless otherwise indicated. In general, 50%–90% of the nuclei were intact and capable of transport. In nuclei with two or more lobes, both lobes accumulated nuclear proteins equally well. (Any possible nonspecific binding to nuclei, nuclear envelopes, or nuclear pores was effectively competed away by the high concentrations of protein and membrane present in the Xenopus egg extract.) Densitometric scanning of photographic negatives to quantitate accumulation of fluorescent nuclear protein was performed as described in Newmeyer et al. (1986b).

Preparation of Peptide-Protein Conjugates

Synthetic peptides were synthesized by the University of California (San Diego) Peptide-Oligonucleotide Synthesis Facility. They were coupled to HSA (Calbiochem) using the heterobifunctional crosslinker MBS, as described by Goldfarb et al. (1986). The average coupling ratio (average number of peptides attached to each molecule of HSA) was measured by SDS gel electrophoresis. The coupling ratio was taken as the difference in apparent molecular weight between the conjugate and MBS-treated HSA, divided by the molecular weight of the peptide. Conjugates with different coupling ratios could be obtained by varying the amount of peptide added from 0.5–5.0 mg per 10 mg HSA in the coupling reaction. Unless otherwise indicated, conjugates with the highest coupling ratio (20 for wt-HSA and 18 for mutant-HSA) were used for all experiments.

Preparation of Protein-Colloidal Gold Complexes

Colloidal gold particles (10 nm; Janssen) were coated with nucleoplasmin using the method of Feldherr et al. (1984), except that nucleoplasmin was isolated from eggs (Newmeyer et al., 1986a) rather than oocytes, and no ethanol extraction of nucleoplasmin was performed. Colloidal gold probes were prepared on the day of the experiment, since they formed aggregates on storage. HSA-peptide conjugates were complexed with gold essentially in the same way, but with certain modifications. When added in excess, HSA-peptide conjugates were found to precipitate colloidal gold particles at neutral pH, apparently because of the number of basic amino acid residues. The conjugates were therefore dialyzed against 10 mM sodium carbonate (pH 11.0) and the colloidal gold solution was adjusted to pH 10.5 using dilute NaOH before mixing. Approximately 0.1 mg of conjugate was used to stabilize 5 ml of colloid. Upon ultracentrifugation, the colloid was recovered in ~50 μl. Usually 0.1 vol or 0.2 vol of colloidal gold solution (in 10 mM sodium carbonate, [pH 11.0]) was added to 1 vol of egg extract; this caused neither a significant change in pH of the extract nor precipitation of the gold particles. In experiments where three to four times greater amounts of conjugate-coated gold particles were used, the amount of protein used was 0.3–0.4 mg/ml of each conjugate as protein.

Electron Microscopic Assay for Transport

Rat liver nuclei (3000/μl) were added to 50–100 μl aliquots of egg extract and allowed to equilibrate for 30 min. After a further incubation with or without the addition of apyrase (final concentration 100 U/ml), hexokinase (100 U/ml) plus 10 mM glucose (Newmeyer et al., 1986a, 1986b), or WGA (final concentration 1.0 mg/ml), solutions of protein-complexed colloidal gold particles were then added. After 30 min, samples were diluted to 1.0 ml with 0.2 M sodium cacodylate (pH 7.4), and within 15 sec, glutaraldehyde was added to 2.5% from a 50% stock solution. After 2 hr on ice, the samples were centrifuged for 30 sec in an Eppendorf centrifuge. The pellet was dispersed in 1.0 ml of 0.2 M sodium cacodylate (pH 7.4) and recentrifuged. The resulting pellet was broken up gently with a micropipet tip, embedded in 2% low-gelling-temperature agarose (SeaKem) in the same buffer, and post-fixed in OsO_4 for 1 hr at 4°C. Samples were dehydrated in a graded ethanol series, embedded in Spurr's resin, sectioned, poststained with uranyl acetate and lead citrate, and examined using a Philips EM 300 electron microscope at 80 kV with a 50 mm objective aperture.

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References

Berrios, M., and Fisher, P. (1986). A myosin heavy chain-like polypeptide is associated with the nuclear envelope in higher eukaryotic cells. J. Cell Biol. *103*, 711–724.

Blow, J. J., and Laskey, R. A. (1986). Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of Xenopus eggs. Cell *47*, 577–587.

Bonner, W. M. (1975a). 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*, 421–430.

Bonner, W. M. (1975b). Protein migration into nuclei. II. Frog oocyte nuclei accumulate a class of microinjected oocyte nuclear proteins and exclude a class of microinjected oocyte cytoplasmic proteins. J. Cell Biol. *64*, 431–437.

Bonner, W. M. (1978). Protein migration and accumulation in nuclei. In The Cell Nucleus, Vol. 6, Part C, H. Busch, ed., (New York: Academic Press), pp. 97–148.

Bürglin, T. R., and De Robertis, E. M. (1987). The nuclear migration signal of Xenopus laevis nucleoplasmin. EMBO J., *6*, 2617–2625.

Bürglin, T. R., Mattaj, I. W., Newmeyer, D. D., Zeller, R., and De Robertis, E. M. (1987). Cloning of nucleoplasmin from Xenopus laevis oocytes and analysis of its developmental expression. Genes Dev. *1*, 97–107.

Davey, J., Dimmock, N. J., and Colman, A. (1985). Identification of the sequence responsible for the nuclear accumulation of the influenza virus nucleoprotein in Xenopus oocytes. Cell *40*, 667–675.

Davis, L. I., and Blobel, G. (1986). Identification and characterization of a nuclear pore complex protein. Cell *45*, 699–709.

De Robertis, E. M. (1983). Nucleocytoplasmic segregation of proteins and RNAs. Cell *32*, 1021–1025.

De Robertis, E. M., Longthorne, R. F., and Gurdon, J. B. (1978). Intracellular migration of nuclear proteins in Xenopus oocytes. Nature *272*, 254–256.

Dingwall, C., and Laskey, R. A. (1986). Protein import into the cell nucleus. Annu. Rev. Cell Biol. *2*, 367–390.

Dingwall, C., Sharnick, S. V., and Laskey, R. A. (1982). A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. Cell *30*, 449–458.

Dingwall, C., Dilworth, S. M., Black, S. J., Kearsey, S. E., Cox, L. S., and Laskey, R. A. (1987). Nucleoplasmin cDNA sequence reveals polyglutamic acid tracts and a cluster of sequences homologous to putative nuclear localization signals. EMBO J. *6*, 69–74.

Dreyer, C., Stick, R., and Hausen, P. (1986). Uptake of oocyte nuclear proteins by nuclei of Xenopus embryos. In Nucleocytoplasmic Transport, R. Peters and M. Trendelenburg, eds. (Heidelberg: Springer Verlag), pp. 143–157.

Feldherr, C. M., Kallenbach, E., and Schultz, N. (1984). Movement of a karyophilic protein through the nuclear pores of oocytes. J. Cell Biol. *99*, 2216–2222.

Finlay, D. R, Newmeyer, D. D., Price, T. M., and Forbes, D. J. (1987). Inhibition of in vitro nuclear transport by a lectin that binds to nuclear pores. J. Cell Biol. *104*, 189–200.

Forbes, D. J., Kirschner, M. W., and Newport, J. W. (1983). Spontaneous formation of nucleus-like structures around bacteriophage DNA microinjected into Xenopus eggs. Cell *34*, 13–23.

Franke, W. W. (1974). Structure, biochemistry, and functions of the nuclear envelope. Int. Rev. Cytol. *4*, 71–236.

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

Gurdon, J. B. (1970). Nuclear transplantation and the control of gene activity in animal development. Proc. Roy. Soc. (London) B *176*, 303–314.

Hall, M. N., Hereford, L., and Herskowitz, I. (1984). Targeting of E. coli β-galactosidase to the nucleus in yeast. Cell *36*, 1057–1065.

Hanover, J. A., Cohen, C. K., Willingham, M. C., and Park, M. K. (1987). O-linked N-acetylglucosamine is attached to proteins of the nuclear pore: evidence for cytoplasmic glycosylation. J. Biol. Chem. *262*, 9887–9894.

Holt, G. D., and Hart, G. W. (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.

Holt, G. D., Snow, C. M., Senior, A., Haltiwanger, R. S., Gerace, L., and Hart, G. W. (1987). Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked N-acetylglucosamine. J. Cell Biol. *104*, 1157–1164.

Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984a). A short amino acid sequence able to specify nuclear location. Cell *39*, 499–509.

Kalderon, D., Richardson, W. D., Markham, A. F., and Smith, A. E. (1984b). Sequence requirements for nuclear location of simian virus 40 large-T antigen. Nature *311*, 499–509.

Krippl, B., Ferguson, B., Jones, N., Rosenberg, M., and Westphal, H. (1985). Mapping of functional domains in adenovirus E1A proteins. Proc. Natl. Acad. Sci. USA *82*, 7480–7484.

Krohne, G., and Franke, W. W. (1980). Immunological identification and localization of the predominant nuclear protein of the amphibian oocyte nucleus. Proc. Natl. Acad. Sci. USA *77*, 1034–1038.

Lanford, R. E., and Butel, J. S. (1984). Construction and characterization of an SV40 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 SV40 T antigen transport signal. Cell *46*, 575–582.

Lang, I., Scholz, M., and Peters, R. (1986). Molecular mobility and nucleocytoplasmic flux in hepatoma cells. J. Cell Biol. *102*, 1183–1190.

Laskey, R. A., Gurdon, J. B., and Trendelenburg, M. (1979). Accumulation of materials involved in rapid chromosomal replication in early amphibian development. Brit. Soc. Dev. Biol. Symp. *4*, 65–80.

Lohka, M. J., and Masui, Y. (1983). Formation in vitro of sperm pronuclei and mitotic chromosomes by amphibian ooplasmic components. Science *220*, 719–721.

Lohka, M. J., and Masui, Y. (1984). Roles of cytosol and cytoplasmic particles in nuclear envelope assembly and sperm pronuclear formation in cell-free preparations from amphibian eggs. J. Cell Biol. *98*, 1222–1230.

Maul, G. G. (1977). The nuclear and cytoplasmic pore complex: structure, dynamics, distribution, and evolution. Int. Rev. Cytol. *6*, (Suppl.) 75–186.

Moreland, R. B., Nam, H. G., Hereford, L. M., and Fried, H. M. (1985). Identification of a nuclear localization signal of a yeast ribosomal protein. Proc. Natl. Acad. Sci. USA *82*, 6561–6565.

Newmeyer, D. D., Lucocq, J. M., Bürglin, 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*, 501–510.

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.

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

Newport, J., and Forbes, D. J. (1985). Fate of DNA injected into Xenopus eggs and in egg extracts: assembly into nuclei. Banbury Report 20: Genetic Manipulation of the Early Mammalian Embryo, (Cold Spring Harbor, New York: Cold Spring Harbor Press), pp. 243–250.

Newport, J., and Forbes, D. J. (1987). The nucleus: structure, function and dynamics. Annu. Rev. Biochem. *56*, 535–565.

Newport, J., and Spann, T. (1987). Disassembly of the nucleus in mitotic extracts: membrane vesicularization, lamin disassembly, and chromosome condensation are independent processes. Cell *48*, 219–230.

Paine, P. L., and Horowitz, S. B. (1980). The movement of material between nucleus and cytoplasm. Cell Biol. *4*, 299–338.

Park, M. K., D'Onofrio, M., Willingham, M. C., and Hanover, J. A. (1987). A monoclonal antibody against a family of nuclear pore proteins (nucleoporins) recognizes a shared determinant: O-linked N-acetylglucosamine. Proc. Natl. Acad. Sci. USA *84*, 6462–6466..

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

Peters, R., Lang, I., Scholz, M., Schulz, B., and Kayne, F. (1986).

Fluorescence microphotolysis to measure nucleocytoplasmic transport in vivo et vitro. Biochem. Soc. Trans. (Lond.) *14*, 821–822.

Richardson, W. D., Roberts, B. L., and Smith, A. E. (1986). Nuclear location signals in polyoma virus large-T. Cell *44*, 77–85.

Richter, J. D., Young, P., Jones, N. C., Krippl, B., Rosenberg, M., and Ferguson, B. (1985). A first exon-encoded domain of E1A sufficient for post-translational modification, nuclear-localization, and induction of adenovirus E3 promoter expression in Xenopus oocytes Proc. Natl. Acad. Sci. USA *82*, 8434–8438.

Scheer, U., and Franke, W. W. (1969). Negative staining and adenosine triphosphatase activity of annulate lamellae of newt oocytes. J. Cell Biol. *42*, 519–533.

Schindler, M., Hogan, M., Miller, R., and DeGaetano, D. (1987). A nuclear specific glycoprotein representative of a unique pattern of glycosylation. J. Biol. Chem. *262*, 1254–1260.

Schröder, H. C., Rottman, M., Bachmann, M., and Müller, W. E. G. (1986). Purification and characterization of the major nucleoside triphosphatase from rat liver nuclear envelopes. J. Biol. Chem. *261*, 663–668.

Schwartzmann, G. O. H., Weigandt, H., Rose, B., Zimmerman, A., Ben-Haim, D., and Loewenstein, W. R. (1981). The diameter of the cell-to-cell junctional membrane channels, as probed with neutral molecules. Science *213*, 551–553.

Silver, P. A., Keean, L. P., and Ptashne, M. (1984). Amino terminus of the yeast GAL4 gene product is sufficient for nuclear localization, Proc. Natl. Acad. Sci. USA *81*, 5951–5955.

Snow, C. M., Senior, A., and Gerace, L. (1987). Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. J. Cell Biol. *104*, 1143–1156.

Unwin, P. N. T., and Milligan, R. A. (1982). A large particle associated with the perimeter of the nuclear pore complex. J. Cell Biol. *93*, 63–75.

Vale, R. D., Reese, T. S., and Sheetz, M. P. (1985). Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. Cell *42*, 39–50.

Wright, C. S. (1977). The crystal structure of wheat germ agglutinin at 2.2 Å resolution. J. Mol. Biol. *111*, 439–457.

Wychowski, C., Benichou, D., and Girard, M. (1986). A domain of SV40 capsid polypeptide VP1 that specifies migration into the cell nucleus. EMBO J. *5*, 2569–2576.

Yasuzumi, G., and Tsubo, I. (1966). The fine structure of nuclei as revealed by electron microscopy. III. Adenosine triphosphatase activity in the pores of nuclear envelope of mouse choroid plexus epithelial cells. Exp. Cell Res. *43*, 281–292.

Note Added in Proof

Recently it has been found that WGA, when injected into cultured cells, has no effect on the ability of 10–17 kd dextrans to diffuse into the nucleus, again arguing that WGA does not act by occluding the pore channel (Yoneda et al., Exp. Cell Res. *173*, 586–595, 1987; Dabauvalle et al., Exp. Cell Res. *174*, 291–296, 1988). WGA did prevent the entry of nucleoplasmin into the nucleus in these experiments.