Evidence for an inhibitory feedback loop regulating simian virus 40 large T-antigen fusion protein nuclear transport

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Nuclear protein import is central to eukaryotic cell function. It is dependent on ATP, temperature and cytosolic factors, and requires specific targeting sequences called nuclear localization signals (NLSs). Nuclear import kinetics was studied *in vitro* using digitonin-permeabilized cells of the HTC rat hepatoma cell line and a fluorescently labelled β -galactosidase fusion protein carrying amino acids 111–135 of the simian virus 40 large T-antigen (T-ag), including the NLS. Nuclear accumulation was rapid, reaching steady-state after about 80 min at 37 °C ($t_{1/2}$ at about 17 min). Surprisingly, maximal nuclear concentration was found to be directly proportional to the concentration of the cytosolic extract and of cytoplasmic T-ag protein. Neither preincubation of cells for 1 h at 37 °C before the addition of T-ag protein nor the addition of fresh transport medium after 1 h and continuation

of the incubation for another hour affected the maximal nuclear concentration. If cells were allowed to accumulate T-ag protein for 1 h before the addition of fresh transport medium containing different concentrations of T-ag protein and incubated for a further hour, the maximal nuclear concentration did not change unless the concentration of T-ag protein in the second transport mixture exceeded that in the first, in which case the nuclear concentration increased. Nuclear import of T-ag thus appeared (i) to be strictly unidirectional over 2 h at 37 °C and (ii) to be regulated by an inhibitory feedback loop, whereby the cytosolic concentration of protein appears to determine directly the precise end point of nuclear accumulation. This study represents the first characterization of this previously undescribed mechanism of regulation of nuclear protein import.

INTRODUCTION

The fact that eukaryotic cells possess a nucleus (karyon) means that the genetic material, the DNA, is separated by the nuclear envelope from the site of protein synthesis, the cytoplasm. Specific transport in both directions between the nucleus and cytoplasm is required for cell function. The exchange of ions, molecules, proteins, RNA and DNA between the cytosol and the nucleus occurs through the nuclear pore complex (NPC) integrated into the nuclear envelope [1–3]. The NPC is a large supramolecular structure of about 125 MDa mass and appears to harbour several exchange pathways which are functionally different [1–3]. Ions and molecules up to a radius of about 4.5 nm are apparently freely exchanged between the nuclear and cytoplasmic compartments [4,5]. Larger molecules, however, including most protein and nucleic acid molecules, are transported through the NPC by an active transport mechanism which is highly selective and requires ATP [6] and specific targeting sequences, called nuclear localization signals (NLSs) [7]. Cytosolic factors are also required, although the transport of nucleic acids appears to require factors distinct from those required for nuclear protein import [8,9]. Transport into and out of the nucleus can be blocked by antibodies to NPC components [10], underlining the central role of the NPC in nucleocytoplasmic transport processes.

NLSs are defined as the sequences sufficient and necessary for nuclear localization of their respective proteins. They have been identified in a number of proteins [7], and are capable of directing normally cytosolic carrier proteins such as β -galactosidase to the nucleus [11]. Several putative receptors for specific NLSs called NLS-binding proteins (NLSBPs) have been identified by their

ability to bind NLSs specifically [12]. Interestingly, they appear to be cytosolic proteins and not an integral part of the NPC. A number of other cytosolic components playing a role in nuclear protein import have recently been identified and defined at the molecular level [13–18].

Two steps of the nuclear import process have been defined [19]. First, NLS-carrying transport substrate binds to an NLSBP binding complex including karyopherin α /importin 60 and karyopherin β /importin 90 which mediates docking to particular components of the NPC [16–18]. The nuclear protein is subsequently translocated through the NPC into the nucleus in ATP-dependent fashion, mediated by the monomeric GTP-binding protein Ran/T4 [13] and its activating protein p10 [14]; other Ran/TC4 interacting proteins such as RCC1 and RanGAP1 may also be involved (see ref. [14]). Nuclear transport appears to be unidirectional in the case of most proteins, i.e. they are retained within the nucleus at high concentrations relative to the cytosol until the nuclear envelope is disrupted during mitosis [20,21]. However, others appear to be able to shuttle between the cytosol and the nucleus, either constitutively or in response to regulatory stimuli [22–24]. The rate of transport is directly correlated to the number of NLSs per protein and indirectly correlated to the mass of the protein [21,25]. In addition, phosphorylation can influence the kinetics of nuclear import of proteins in a cell-cycle-dependent or signal-transduction responsive manner [26], examples being the simian virus 40 large tumour antigen (T-ag) [27–30], the yeast transcription factor SW15 [31,32], the *Drosophila* morphogen dorsal [33], the *Xenopus laevis* nuclear protein nucleoplasmin [34] and the protooncogene *c-rel* [35].

This study examines the nuclear import kinetics of a T-ag

Abbreviations used: NLS, nuclear localization signal; NLSBP, NLS-binding protein; T-ag, simian virus 40 large T-antigen; NPC, nuclear pore complex; DTT, dithiothreitol; IAF, 5-iodoacetamidofluorescein; CLSM, confocal laser scanning microscopy.

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fusion protein *in vitro*, examining the concentration-dependence of the maximal level of nuclear accumulation. We show that T-ag nuclear import is unidirectional, and that the cytosolic concentration of protein appears to determine the maximal level of nuclear accumulation precisely. The results provide the first direct evidence for an inhibitory feedback loop which regulates protein transport into the nucleus by determining the maximal level of nuclear accumulation at steady-state.

MATERIALS AND METHODS

Fusion protein expression and fluorescent labelling

A T-ag β -galactosidase fusion protein, T-ag(WT 111–135) [27], in which T-ag amino acids 111–135 are fused N-terminal to the *E. coli* β -galactosidase enzyme (amino acids 9–1023), was used as the nuclear import substrate. The protein was expressed in *E. coli* and purified by affinity chromatography as described [27–30], before dialysis in 50 mM bicine/KOH, pH 7.8, containing 150 mM NaCl. The protein concentration (measured using the Bradford dye-binding assay [36] with BSA as standard) was then adjusted to 10 mg/ml, and 50 μ l aliquots were stored at -70°C . For labelling, an aliquot was supplemented with dithiothreitol (DTT; final concentration 5 mM) and incubated for 1 h at 37°C . DTT was then removed from the reduced T-ag(WT 111–135) using a centricon (Amicon; 100 kDa molecular-mass cut-off), and then 2.5 μ l of 200 mM 5-iodoacetamidofluorescein (IAF; Molecular Probes) in DMSO were added to give a final concentration of 10 mM IAF (a molar ratio of IAF to protein of 1:100). Incubation was for 1 h at room temperature in the dark, after which 50 μ l of 50 mM bicine/KOH, pH 7.8, and 2.5 μ l of 200 mM IAF were added and the incubation continued for another hour. The mixture was then passed over a Sephadex G-25 column run in transport buffer (50 mM Hepes/KOH, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT). Fractions of about 700 μ l were collected and protein-containing peak fractions pooled and concentrated before storage in 10 μ l aliquots at -70°C .

Cell culture

Cells of the HTC cell line, a derivative of Morris hepatoma 7288C (Flow Laboratories), were cultivated as described previously [27–30]. For transport experiments, cells were seeded on to sterile 15 mm $\times$ 15 mm glass slides 48 h before use.

In vitro transport assay

A modification of the digitonin permeabilization method of Adam et al. [37], which results in permeabilization of the cell membrane but leaves the nuclear envelope intact, was used. The cytosolic extract used was rabbit reticulocyte lysate (Promega) previously dialysed overnight at 4°C against a transport buffer supplemented with 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin and 1 $\mu\text{g}/\text{ml}$ aprotinin. Collodion membranes (Schleicher & Schüll) with a cut-off of 10 kDa were used for vacuum dialysis, subsequent to which the protein concentration was adjusted to either 40 or 120 mg/ml and the lysate stored in 20 μ l aliquots at -70°C .

The final concentrations in the nuclear transport assay were 0–96 mg/ml reticulocyte lysate, 2 mM ATP, 10 mM phosphocreatine, 80 units/ml creatine phosphokinase and either 40–1000 $\mu\text{g}/\text{ml}$ T-ag(WT 111–135) or 24 $\mu\text{g}/\text{ml}$ fluorescently labelled dextran (70 kDa; Sigma Chemical Co.). Phosphocreatine and ATP were prepared freshly for each experiment. Creatine phosphokinase was stored in 10 μ l aliquots at -20°C . The

permeabilization of cells grown on coverslips was performed by immersing them in 2 ml of 50 $\mu\text{g}/\text{ml}$ digitonin (Calbiochem) for 5 min on ice. The coverslip was then washed three times with 1 ml of ice-cold transport buffer and used immediately. A 5 μ l droplet of transport mixture was pipetted into a chamber on a glass slide constructed with Scotch 3M pressure-sensitive double-sided tape (0.2 mm thick) into which a circular hole had been punched (approx. 5 mm diameter). The coverslip was placed cell-side face-down on to the drop in the chamber and sealed with nail polish. Cells were then incubated at 37°C for various times as indicated. In experiments in which the transport mixture was changed after 1 h, a 25 μ l droplet of transport mixture was pipetted on to a sheet of parafilm and the coverslip placed cell-side face-down on to the droplet. After the incubation at 37°C in a wet chamber, the coverslip was carefully removed, washed once with 1 ml of ice-cold transport buffer and then mounted as described above, before incubation at 37°C .

Quantification of nuclear transport efficiency

The quantification of nuclear import was performed by confocal laser scanning microscopy (CLSM) as described previously [27–30], using a $40\times$ phase-contrast lens (NA 1.3; oil immersion; Nikon). The laser beam power (488 nm line of an argon/krypton laser) was adjusted such that photobleaching was negligible, and the confocal aperture adjusted to be smaller than the average cross-section of the nuclei in the z -direction. The hallmark of CLSM is its ability to eliminate virtually all fluorescence light from out-of-focus areas. Thus light is collected only from a well-defined optical section and its intensity can be correlated directly with the concentration of a fluorophore. This feature allows measurement of nuclear fluorescence directly without removing the transport reaction mixture which contains fluorescently labelled protein or dextran [27,38]. Within one field of view (512–512 pixels) the pixel intensity (0–255 units) was determined inside all nuclei and in the cytosol ('medium') as close as possible to the nucleus, employing a software function. As observed using other systems [29,39], diffusion from medium to the cytoplasm of permeabilized cells was essentially instantaneous (and hence not a limiting factor in terms of nuclear import). This is supported by the fact that in all cases where measured, medium fluorescence was essentially identical with that in the cytoplasm.

The relative nuclear and cytoplasmic fluorescence was routinely corrected for the dark current by subtracting its pixel intensity [27–30,39,40]. From the corrected pixel intensities for the nuclear and medium measurements, the mean pixel intensities F_n and F_c were calculated respectively, which were then used for the calculation of the ratio of nuclear to cytosolic fluorescence, $F_{(n/c)}$. As observed previously [29,39,40], the fact that the volume of the medium vastly exceeds that of the accumulating nuclei means that the medium cannot be depleted of transport substrate and F_c remains constant during the course of an experiment; this was also routinely confirmed by direct measurements. When no fluorescently labelled fusion protein was in the medium, F_c of a control experiment was used. Unless otherwise indicated, $F_{(n/c)}$ results were routinely expressed as a percentage of the $F_{(n/c)}$ for the standard experimental conditions of 200 $\mu\text{g}/\text{ml}$ T-ag(WT 111–135) and 32 mg/ml cytosol at 2 h at 37°C . For time-course experiments, the data were fitted using the function:

$$F_{(n/c)}(t) = F_{(n/c)}(\infty) \times (1 - e^{-tk})$$

(see refs [29,30,32]), where $F_{(n/c)}(t)$ is $F_{(n/c)}$ at time t , $F_{(n/c)}(\infty)$ is the extrapolated maximal $F_{(n/c)}$ at infinite time, and k is the rate constant.

RESULTS

Nuclear uptake of a T-ag- β -galactosidase fusion protein *in vitro*

The regulation of protein import into the nucleus was studied using a fluorescently labelled T-ag-NLS-containing fusion protein T-ag(WT 111–135) [27] as a substrate and an *in vitro* assay based on digitonin-permeabilized HTC cells [37]. Quantification was performed using CLSM, which we have previously used in conjunction with microinjection [27–30,32]. Although the digitonin-permeabilized cell system has been used successfully to examine the nuclear transport of protein–peptide conjugates

Figure 1 Visualization of nuclear accumulation of T-ag fusion protein *in vitro*

(Upper panel) Dependence of active transport on temperature and ATP. HTC cells were permeabilized with digitonin and incubated with cytosolic extract and 200 μ g/ml T-ag(WT 111–135) (A) in the presence of an ATP-regenerating system at 37 °C and (B) in the absence of an ATP-regenerating system at 4 °C for 60 min. CLSM scans were taken with identical settings, and are shown as grey scale images, where black relates to the lowest pixel intensity and white to the highest. (Lower panel) Time course of nuclear import. HTC cells were permeabilized with digitonin and incubated with cytosolic extract and 200 μ g/ml T-ag(WT 111–135); CLSM scans were taken after 5 (A), 20 (B), 40 (C) and 100 (D) min with identical settings. It should be noted that the images in the upper panel were scanned with a higher photomultiplier gain than those here, in order to facilitate visualization of the ring staining in the upper panel, part B; the nuclei in the upper panel, part A, are slightly overexposed as a result.

Table 1 Dependence of nuclear import on the presence of cytosolic extract and an ATP-regenerating system

Permeabilized HTC cells were incubated with T-ag(WT 111–135) for 1 h at 37 °C with or without cytosolic extract (32 mg/ml) and in the presence or absence of an ATP-regenerating system. $F_{(n/c)}$ (fluorescence quantified in the nucleus, F_n , relative to that quantified in the cytoplasm, F_c) was calculated as described in the Materials and methods section. Results for $F_{(n/c)}$ are means $\pm$ S.D. for at least 79 nuclei, and expressed as a percentage of T-ag(WT 111–135) in the presence of cytosol and ATP-regenerating system.

	Cytosol	ATP-regenerating system	Parameter of nuclear transport*	
			Mean $F_{(n/c)}$	(%)
T-ag(WT 111–135)	Present	Present	11.21 ± 0.29	100
T-ag(WT 111–135)	Absent	Present	$1.52 \pm 0.35^*$	14
T-ag(WT 111–135)	Present	Absent	$1.30 \pm 0.29^*$	12
T-ag(WT 111–135)	Absent	Absent	$1.18 \pm 0.45^*$	11

* The relatively high standard deviation in these samples where accumulation is low due to the lack of cytosol and/or ATP-regenerating system is attributable in part to variations in the efficiency of the fluorescence measurement at low signal-to-noise ratios (see also ref. [38]).

[37,41–44], we intended to use larger transport substrates with more physiological NLS to mass ratios (about 1:119000 compared with 1:6700 for a BSA–peptide conjugate bearing 10 NLS peptides), and hence it was initially necessary to test if T-ag(WT 111–135) would be transported to the nucleus by digitonin-permeabilized HTC cells.

In the presence of cytosolic extract and an ATP-regenerating system, strong nuclear accumulation of T-ag(WT 111–135) was observed (Figure 1, upper panel, part A). This was in stark contrast with the lack of import when the incubation was performed without ATP at 4 °C (Figure 1, upper panel, part B). In this case, T-ag(WT 111–135) localized to the nuclear envelope, implying that docking but not translocation into the nucleus was occurring. Import in the presence of cytosolic extract but in the absence of the ATP-regenerating system was negligible (Table 1). Nuclear accumulation of T-ag(WT 111–135) at 37 °C in the presence of ATP and cytosol was rapid up until about 40 min (Figure 1, lower panel, parts A–C), before slowing and ultimately reaching steady-state well before 2 h (Figure 1, lower panel, part D). In the presence of cytosol and the ATP-regenerating system, a 70 kDa dextran (exceeding the molecular-mass cut-off of about 45 kDa for passive diffusion into the nucleus [1–3]) was excluded from the nucleus [maximal $F_{(n/c)}$ of 0.13, compared with that of 11.21 for T-ag(WT 111–135); see Table 1], indicating the intactness of the nuclei of digitonin-permeabilized cells.

Dependence of nuclear import kinetics on the concentration of exogenous cytosol and transport substrate

The kinetics of this process were investigated in detail in transport experiments in which the concentration of either cytosolic extract or T-ag(WT 111–135) substrate was varied. In the case of the former, 200 μ g/ml T-ag(WT 111–135) was used together with various concentrations of cytosolic extract (Figure 2A). Maximal accumulation was attained after about 80 min, $t_{1/2}$ being very similar for the different concentrations of cytosolic extract. Interestingly, the maximal nuclear concentration of T-ag(WT 111–135) appeared to correlate directly with the concentration of the cytosolic extract, showing a linear relationship (Figure 2B).

Figure 2 Dependence of nuclear import on the concentration of cytosolic extract

Permeabilized HTC cells were incubated with 200 $\mu\text{g/ml}$ T-ag(WT 111–135) and various concentrations of cytosolic extract as indicated at 37 °C. The time course (min) of nuclear accumulation (A) and the relationship between maximal nuclear accumulation and the protein concentration of the cytosolic extract (B) are shown. (A) The incubation was carried out with 16 (Δ), 32 ($\bullet$) and 64 ($\circ$) mg/ml cytosolic extract. At the time points indicated the relative nuclear and cytosolic fluorescence was measured and the $F_{\text{in/c}}$ of T-ag(WT 111–135) calculated. The mean $F_{\text{in/c}}$ (at least 33 nuclei), expressed as a percentage of the maximal nuclear accumulation at infinite time achieved using 32 mg/ml cytosolic extract, was plotted against time and curves fitted as described in the Material and methods section. (B) Nuclear accumulation, expressed as a percentage of the nuclear accumulation at infinite time achieved using 32 mg/ml cytosolic extract (A), is plotted against the protein concentration of the cytosolic extract. The result of two separate experiments is shown. Each value is the mean for greater than 73 (or 19 in the inset) nuclei, with the standard deviation shown as error bars.

When the concentration of cytosolic extract was below 16 mg/ml, nuclear import was not significantly higher than that in the absence of cytosol (Figure 2B, inset). That a low amount of nuclear transport occurred in the absence of cytosol (see Table 1) presumably results from the fact that residual cell-associated cytosol (and ATP) remain after cell permeabilization with digitonin.

The effect of different concentrations of T-ag(WT 111–135) on the kinetics of nuclear protein import was studied by incubating cells in the presence of 32 mg/ml cytosolic extract (Figure 3). As for cytosolic extract, maximal nuclear accumulation correlated directly with the concentration of T-ag(WT 111–135) in the cytosolic extract, showing a linear relationship (Figure 3B). The detection limit of the confocal microscope was 40 $\mu\text{g/ml}$ cytosolic

Figure 3 Dependence of nuclear import on the concentration of transport substrate

Permeabilized HTC cells were incubated with 32 mg/ml cytosolic extract and various concentrations of T-ag(WT 111–135) as indicated at 37 °C. The time course of nuclear accumulation (A) and the linear relationship between maximal nuclear accumulation and the transport substrate concentration (B) are shown. (A) The incubation was carried out with 500 (Δ), 200 ($\bullet$; average of two experiments) and 40 ($\square$) $\mu\text{g/ml}$ T-ag(WT 111–135). Measurements (greater than 23 nuclei/measurement), expressed as a percentage of the maximal nuclear accumulation at infinite time achieved using 200 $\mu\text{g/ml}$ T-ag(WT 111–135), were performed as described in the legend to Figure 2 (see the Materials and methods section). (B) Nuclear accumulation, expressed as a percentage of the maximal nuclear accumulation at infinite time achieved using 200 $\mu\text{g/ml}$ T-ag(WT 111–135) (see A), is plotted against the concentration of T-ag(WT 111–135). Each value is the mean for greater than 75 nuclei with the standard deviations shown as error bars.

T-ag(WT 111–135). Thus it was not possible to determine the lower threshold of nuclear transport in terms of nuclear import substrate concentration. $t_{1/2}$ was attained about twice as slowly at 500 as at 200 and 40 $\mu\text{g/ml}$ T-ag(WT 111–135) (Figure 3A).

Demonstration that the dependence of maximal nuclear import on the concentration of exogenous cytosol and transport substrate is not due to exhaustion of cytosolic factors

The dependence of maximal nuclear concentration on the concentration of cytosolic extract and also of T-ag fusion protein import substrate implied the existence of a mechanism regulating the maximal level of nuclear protein accumulation. However, it was important to dismiss trivial explanations such as the exhaus-

Table 2 Demonstration that neither exhaustion nor inactivation of cytosolic factors limits the maximal nuclear accumulation of transport substrate

In experiment A transport mixture was preincubated with permeabilized HTC cells for 1 h at 37 °C, and then incubated with freshly permeabilized cells for a further 1 h. As a control, permeabilized cells were incubated with transport mixture for 1 h. In experiment B permeabilized HTC cells were either incubated at 37 °C for 1 h with T-ag(WT 111–135) or preincubated for 1 h with transport mixture without T-ag(WT 111–135), followed by an exchange of transport mixture and continuation of the incubation with T-ag(WT 111–135) for further 1 h. In experiment C permeabilized HTC cells were incubated at 37 °C either continuously for 2 h or for 1 h followed by an exchange of transport mixture and continuation of the incubation for 1 h. Results for $F_{(n/c)}$ (A, at least 103 nuclei; B, at least 84 nuclei; C, at least 25 nuclei) are expressed as a percentage (mean $\pm$ S.D.) relative to the control experiment for each preincubation.

Concn. of cytosolic extract (mg/ml)	Concn. of T-ag(WT 111–135) (μ g/ml)	Preincubation (1 h)	$F_{(n/c)}$ (%)
A. Preincubation of transport mixture with permeabilized HTC cells			
32	200	None (1 h incubation)	100 $\pm$ 10
32	200	Transport mixture with HTC cells	70 $\pm$ 19
B. Preincubation of permeabilized HTC cells with cytosol without transport substrate			
32	200	None (1 h incubation)	100 $\pm$ 10
32	200	HTC cells with transport mixture lacking substrate	86 $\pm$ 19
C. Preincubation of permeabilized HTC cells with transport mixture containing various concentrations of cytosolic extract and transport substrate as indicated			
32	200	None (2 h incubation)	100 $\pm$ 19
32	200	HTC cells with transport mixture	114 $\pm$ 18
32	40	None (2 h incubation)	100 $\pm$ 11
32	40	HTC cells with transport mixture	129 $\pm$ 19
16	200	None (2 h incubation)	100 $\pm$ 13
16	200	HTC cells with transport mixture	90 $\pm$ 14
64	200	None (2 h incubation)	100 $\pm$ 21
64	200	HTC cells with transport mixture	110 $\pm$ 21

tion or inactivation of the cytosolic extract during the time of incubation. This was addressed in several ways: first, the usual transport mixture of cytosol and substrate protein was incubated with permeabilized HTC cells for 1 h, before transfer to another batch of permeabilized HTC cells and incubation for a further 1 h (Table 2A). Nuclear accumulation during the second incubation with the same cytosolic extract and substrate protein indicated only minimal loss of transport activity due to the preincubation.

In another experiment, permeabilized HTC cells were incubated with transport mixture without transport substrate for 1 h, after which the transport mixture was replaced by a mixture containing T-ag(WT 111–135), and incubation was continued for 1 h (Table 2B). The maximal nuclear concentration of T-ag(WT 111–135) did not differ significantly from that of cells incubated for 1 h without prior incubation with cytosol. It thus appeared that the nuclear transport system did not become inactivated over the 1 h incubation nor was the cytosolic extract able to saturate the nuclei with nuclear transport substrate.

A further series of experiments were performed, in which incubations were performed with transport mixture containing various combinations of concentrations of T-ag(WT 111–135) and cytosolic extract (Table 2C). Maximal nuclear concentration was determined after either continuous incubation for 2 h or two

Table 3 Unidirectionality of nuclear import as shown in preincubation experiments with various transport-substrate concentrations

Permeabilized HTC cells were incubated as indicated for 1 h at 37 °C with transport mixture containing 32 mg/ml cytosolic extract and 200 (A) or 40 (B) μ g/ml T-ag(WT 111–135). The transport mixture was then removed and fresh mixture added containing 32 mg/ml cytosolic extract and various concentrations of T-ag(WT 111–135) as indicated. The incubation was continued for further 1 h. $F_{(n/c)}$ was calculated for at least 31 and 87 nuclei for (A) and (B) respectively as described in the Materials and methods section and is expressed (mean $\pm$ S.D.) as a percentage of the 2 h 200 μ g/ml T-ag(WT 111–135) control.

Concentration of T-ag(WT 111–135) (μ g/ml)		
1st incubation	2nd incubation	$F_{(n/c)}$ (%)
A. Preincubation of cells with 200 μ g/ml T-ag(WT 111–135)		
200 (2 h)	—	100 $\pm$ 18
40 (2 h)	—	27 $\pm$ 11
200 (1 h)	0 (1 h)	106 $\pm$ 19
200 (1 h)	40 (1 h)	108 $\pm$ 15
200 (1 h)	200 (1 h)	110 $\pm$ 14
B. Preincubation of cells with 40 μ g/ml T-ag(WT 111–135)		
200 (2 h)	—	100 $\pm$ 16
40 (2 h)	—	39 $\pm$ 6
40 (1 h)	0 (1 h)	34 $\pm$ 6
40 (1 h)	40 (1 h)	36 $\pm$ 8
40 (1 h)	200 (1 h)	70 $\pm$ 19

consecutive 1 h incubations whereby the transport mixture was replaced after 1 h by fresh transport mixture of identical composition. No significant difference in the maximal nuclear concentration was observed whether the transport mixture was replaced after 1 h or not. It was concluded on the basis of these results that the correlation between maximal nuclear accumulation and the concentration of either cytosolic extract or transport substrate was not attributable to limitations of the *in vitro* assay.

Unidirectionality of nuclear transport *in vitro*

Further experiments were performed in order to characterize the mechanism of regulation of maximal nuclear accumulation. The directionality of nuclear transport was tested by incubating permeabilized HTC cells with 200 μ g/ml T-ag(WT 111–135) for 1 h, before replacement of the transport mixture with a new mixture containing 0, 40 or 200 μ g/ml T-ag(WT 111–135) (Table 3A) and continuation of the incubation for a further 1 h. Results were compared with those for experiments in which permeabilized HTC cells were incubated continuously with 40 or 200 μ g/ml T-ag(WT 111–135) for 2 h. There was no significant change in the maximal nuclear accumulation of T-ag(WT 111–135), suggesting that, at least on the time scale of the experiment, export or efflux of T-ag(WT 111–135) from the nucleus was negligible. This result was confirmed by experiments in which permeabilized HTC cells were first incubated with 40 μ g/ml T-ag(WT 111–135) for 1 h, followed by incubation with 0, 40 or 200 μ g/ml T-ag(WT 111–135) for another hour (Table 3B). Again in control experiments permeabilized HTC cells were incubated continuously with either 200 or 40 μ g/ml T-ag(WT 111–135) for 2 h. No significant change in maximal nuclear accumulation was observed when the concentration of T-ag(WT 111–135) during the second incubation was either 0 or 40 μ g/ml T-ag(WT 111–135). The nuclear accumulation was almost identical with that of the control experiment with 40 μ g/ml. However, the maximal nuclear

concentration increased significantly when 200 $\mu\text{g/ml}$ T-ag(WT 111–135) was used during the second incubation almost reaching the level of the control experiment with 200 $\mu\text{g/ml}$ T-ag(WT 111–135).

The fact that subsequent to accumulation of transport substrate to near-saturation level (60 min), no export of T-ag(WT 111–135) from the nucleus could be detected up to 60 min after the removal of transport substrate from the cytosolic medium (Table 3A), whereas increasing the medium concentration of transport substrate resulted in increased nuclear uptake, indicated that nuclear transport is uniquely unidirectional in our permeabilized cell system over the time course of the experiment (2 h). This unidirectionality of transport in the import direction is completely consistent with observation in other studies [21,26–30,37–39]. The results also suggest that the concentration of cytosolic transport substrate directly determines the maximal level of accumulation of protein in the nucleus.

DISCUSSION

This study describes some of the basic kinetic characteristics of the nuclear transport of a fusion protein bearing the T-ag NLS. Nuclear import *in vitro* had an exponential nature, reaching saturation after about 80 min; nuclear import *in vivo* of the same transport substrate in microinjected HTC cells similarly shows saturation kinetics [22–24]. Nuclear accumulation after 20 min as well as the maximal level of nuclear accumulation of T-ag fusion protein correlated directly with both the concentration of transport substrate in the medium and the cytosolic extract. Unexpectedly, the maximal level of nuclear accumulation appeared to be dependent on the concentration of both the transport substrate in the medium and the cytosolic extract. This dependence was not attributable to either the exhaustion of components of nuclear import system in the cytosolic extract or their inactivation during the incubation, the maximal level of nuclear accumulation being able to be increased by raising the 'cytosolic' concentration of the transport substrate. The dependence of maximal nuclear accumulation on the cytosolic concentration of transport factors thus most likely reflects a mechanism of specific regulation of nuclear protein transport, rather than an artifact of the *in vitro* system employed.

As indicated by the data of Table 3, subsequent to accumulation to near-saturation levels of T-ag(WT 111–135), no export of transport substrate from the nucleus could be detected up to 60 min after its removal from the cytosolic medium, whereas elevating its medium concentration resulted in renewed/increased nuclear uptake. Nuclear transport can thus be concluded to be unidirectional in the import direction in our permeabilized cell system, consistent with the conclusions based on measurements by others of nuclear protein import *in vitro* [37,39] and *in vivo* [21,26–30,38]; e.g. nuclear-injected T-ag(WT 111–135) or other fusion proteins are not exported from the nuclei of living HTC cells over at least 2 h (U. Seydel and D. A. Jans, unpublished work; see refs [27,38]). It accordingly seems highly unlikely that the maximal nuclear concentration of T-ag is governed by an equilibrium that requires equal rates of import and export (see also refs [27–30]). Rather, a hypothesis more in keeping with the data is that the NLSBP involved – possibly karyopherin α /importin 60, which has been shown to be present in both nucleus and cytoplasm [16] – may shuttle between cytoplasm and nucleus, and thus itself represent the sensory element responsible for regulating the maximal level of nuclear accumulation in accordance with the cytoplasmic levels of substrate. Our results imply that a complex between the transport substrate and at least one factor of the nuclear import system

such as karyopherin/importin, rather than the NLS-containing protein itself, is the actual transport substrate. The concentration-dependence of maximal nuclear accumulation could accordingly be rationalized in terms of the association constant of either the transport substrate with its NLSBP or the substrate–NLSBP complex with components of the NPC. Elevating the concentration of NLSBP in the transport medium has been shown to increase the efficiency of nuclear transport *in vitro* for purified importin 60 [15], as well as to prevent nuclear export of an NLS-peptide–human serum albumin conjugate in the case of karyopherins α and β [45].

Our hypothesis is that the results presented here can be explained by the existence of an inhibitory feedback loop which regulates the maximal level of nuclear accumulation in response to the cytoplasmic concentration of transport substrate. We postulate that a critical cytoplasmic threshold of both transport substrate and the NLSBP exists below which nuclear transport does not take place, which may be indicated by the fact that 16 mg/ml cytosolic extract or less is insufficient to support active nuclear transport, presumably because the amount of NLSBP is insufficient to stimulate transport. Above this threshold, the equilibrium of binding of NLSBP and transport substrate on both sides of the nuclear envelope, which may well be regulated by phosphorylation in the case of T-ag [27–30], determines the final maximal nuclear concentration. Increasing the cytosolic concentration can increase maximal nuclear import because it increases the amount of cytosolic NLSBP, and increasing the cytoplasmic concentration of substrate increases transport presumably by affecting the equilibrium of association of NLSBP and NLS-containing transport substrate in the cytosol. Communication between the cytosol and nucleoplasm is presumably effected by the shuttling NLSBP.

In summary, this study presents evidence for the existence of a putative negative feedback loop regulating nuclear protein import, where the level of maximal nuclear accumulation appears to be determined by the cytosolic concentration of transport substrate. This feedback loop may constitute a third step of nuclear protein import, subsequent to the preceding steps of docking at the nuclear envelope and translocation into the nucleus [13,19,20]. The precise mechanistic role of phosphorylation in mediating the regulation of maximal nuclear accumulation by this negative feedback loop is the focus of future work in this laboratory.

Professor R. Peters is thanked for his support in preliminary studies. This work was supported by a joint grant from the Institute of Advanced Studies Australian Universities' Research Collaboration Scheme, and a grant from the Clive and Vera Ramaciotti Foundation to D.A.J. Patricia Jans is thanked for assistance with tissue culture.

REFERENCES

- Dingwall, C. and Laskey, R. A. (1992) *Science* **258**, 942–948
- Agutter, P. S. and Prochnow, D. (1994) *Biochem. J.* **300**, 609–618
- Pante, N. and Aebi, U. (1994) *Curr. Opin. Struct. Biol.* **4**, 187–196
- Paine, P. L. (1975) *J. Cell Biol.* **66**, 652–657
- Peters, R. (1983) *J. Biol. Chem.* **258**, 11427–11429
- Dingwall, C. and Laskey, R. A. (1986) *Annu. Rev. Cell Biol.* **2**, 367–390
- Boulikas, T. (1993) *Crit. Rev. Eukaryotic Gene Expression* **3**, 193–227
- Forbes, D. J. (1992) *Annu. Rev. Cell Biol.* **8**, 495–527
- Izaurralde, E. and Mattaj, J. W. (1992) *Semin. Cell Biol.* **3**, 279–288
- Featherstone, C., Darby, M. K. and Gerace, L. (1988) *J. Cell Sci.* **107**, 1289–1298
- Kalderon, D., Roberts, B. L., Richardson, W. D. and Smith, A. E. (1984) *Cell* **39**, 499–509
- Yamasaki, L. and Lanford, R. E. (1992) in *Nuclear Trafficking* (Feldherr, C., ed.) pp. 122–174, Academic Press, New York
- Moore, M. S. and Blobel, G. (1993) *Nature (London)* **365**, 661–663
- Moore, M. S. and Blobel, G. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10212–10216

- 15 Görlich, D., Prehn, S., Laskey, R. A. and Hartmann, E. (1994) *Cell* **79**, 767–778
- 16 Morianu, J., Blobel, G. and Radu, A. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 2008–2011
- 17 Radu, A., Blobel, G. and Moore, G. S. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1769–1773
- 18 Görlich, D., Kostka, S., Kraft, R., Dingwall, C., Laskey, R. A., Prehn, S. and Hartmann, E. (1995) *Curr. Biol.* **5**, 383–392
- 19 Newmeyer, D. D. and Forbes, D. J. (1988) *Cell* **52**, 641–653
- 20 Moore, M. S. and Blobel, G. (1992) *Cell* **69**, 939–950
- 21 Dworetzky, S. I., Lanford, R. E. and Feldherr, C. M. (1988) *J. Cell Biol.* **107**, 1279–1287
- 22 Borer, R. A., Lehner, C. F., Eppenberger, H. M. and Nigg, E. A. (1989) *Cell* **56**, 379–390
- 23 Mandell, R. B. and Feldherr, C. M. (1992) *Exp. Cell. Res.* **198**, 164–169
- 24 Hsu, S., Qi, M. and DeFranco, D. B. (1992) *EMBO J.* **11**, 3457–3468
- 25 Goldfarb, D. S., Gariepy, J., Schoolnik, G. and Kornberg, R. D. (1986) *Nature (London)* **322**, 641–644
- 26 Jans, D. A. (1995) *Biochem. J.* **311**, 705–716
- 27 Rihs, H. P. and Peters, R. (1989) *EMBO J.* **8**, 1479–1484
- 28 Rihs, H. P., Jans, D. A., Fan, H. and Peters, R. (1991) *EMBO J.* **10**, 633–639
- 29 Jans, D. A., Ackermann, J. R., Beach, D. H. and Peters, R. (1991) *J. Cell Biol.* **115**, 1203–1212
- 30 Jans, D. A. and Jans, P. (1994) *Oncogene* **9**, 2961–2968
- 31 Moll, T., Tebb, G., Surana, U., Robitsch, H. and Nasmyth, K. (1991) *Cell* **66**, 743–758
- 32 Jans, D. A., Moll, T., Nasmyth, K. and Jans, P. (1995) *J. Biol. Chem.* **270**, 17064–17067
- 33 Norris, J. L. and Manley, J. L. (1992) *Genes Dev.* **6**, 1654–1667
- 34 Vancurova, I., Paine, T. M., Lou, W. and Paine, P. L. (1995) *J. Cell Sci.* **108**, 779–787
- 35 Mosialos, G., Hamer, P., Capobianco, A. J., Laursen, R. A. and Gilmore, T. D. (1991) *Mol. Cell. Biol.* **11**, 5867–5877
- 36 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 37 Adam, S. A., Sterne Marr, R. and Gerace, L. (1990) *J. Cell Biol.* **111**, 807–816
- 38 Peters, R. (1986) *Biochim. Biophys. Acta* **864**, 305–359
- 39 Walaschewski, U., Jans, D. A., Neugebauer-Goedde, D.-C. and Peters, R. (1995) *Methods Mol. Cell. Biol.* **5**, 87–95
- 40 Ymer, S. and Jans, D. A. (1995) *Biotechniques*, in the press
- 41 Adam, S. A., Lobl, T. J., Mitchell, M. A. and Gerace, L. (1989) *Nature (London)* **337**, 276–279
- 42 Adam, S. A. and Gerace, L. (1991) *Cell* **66**, 837–847
- 43 Adam, E. H. and Adam, S. A. (1994) *J. Cell Biol.* **125**, 547–555
- 44 Duverger, E., Pellerin-Mendes, C., Mayer, R., Roche, A.-C. and Monsigny, M. (1995) *J. Cell Sci.* **108**, 1325–1332
- 45 Morianu, J. and Blobel, G. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4318–4322