

# Inhibition of Nuclear Protein Import by Nonhydrolyzable Analogues of GTP and Identification of the Small GTPase Ran/TC4 as an Essential Transport Factor

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**Abstract.** We have investigated a possible involvement of GTPases in nuclear protein import using an in vitro transport system involving digitonin-permeabilized cells supplemented with exogenous cytosol. Transport in this system was measured with a novel ELISA-based assay that allows rapid quantitative analysis. GTP $\gamma$ S and other nonhydrolyzable analogues of GTP were found to rapidly inhibit the rate of in vitro nuclear import. Transport inhibition by GTP $\gamma$ S was dependent on the concentrations of permeabilized cells and cytosol, and was strongly enhanced by a cytosolic factor(s). The predominant cytosolic component responsible for this inhibition was found in a 20–30-kD fraction in molecular sieving chromatography.

Furthermore, a component(s) of this 20–30-kD fraction was itself required for efficient nuclear import. Biochemical complementation with bacterially expressed protein demonstrated that this essential GTP $\gamma$ S-sensitive transport factor was Ran/TC4, a previously described GTPase of the Ras superfamily found in both nucleus and cytoplasm. Ran/TC4 and its guanine nucleotide release protein RCC1 have previously been implicated in DNA replication, cell cycle checkpoint control, and RNA synthesis, processing and export. Our results suggest that Ran/TC4 serves to integrate nuclear protein import with these other nuclear activities.

**M**OLECULAR exchanges between nucleus and cytoplasm occur continuously in eukaryotic cells and are fundamentally important for cellular metabolism. The boundary of the nucleus is formed by the nuclear envelope (NE)<sup>1</sup>, a double membrane system that is continuous with the endoplasmic reticulum. Transport across the NE is carried out by elaborate supramolecular structures called nuclear pore complexes (NPCs; reviewed by Nigg et al., 1991; Forbes, 1992; Gerace, 1992). The NPC has a mass of  $\sim 125 \times 10^6$  D (Reichelt et al., 1990), but relatively few of its polypeptides have been identified. Nevertheless, general transport properties of the NPC have been determined by functional studies. The NPC contains an aqueous channel(s) with a diameter of  $\sim 10$  nm, which allows rapid, nonselective diffusion of ions, metabolites and other small molecules across the NE (Paine et al., 1975; Peters, 1986).

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1. *Abbreviations used in this paper:* ARF, ADP-ribosylation factor; B-BSA, biotinylated BSA; GAP, GTPase-activating protein; GNRP, guanine nucleotide release protein; NE, nuclear envelope; NPC, nuclear pore complex.

Most proteins and RNAs are too large to diffuse through the NPC at physiologically significant rates, and instead are transported through a gated channel in the NPC by highly selective, signal-mediated mechanisms (Nigg et al., 1991; Forbes, 1992; Gerace, 1992).

Signals that specify nuclear protein import have been characterized in detail, and usually involve short basic stretches of amino acids in the transported proteins called nuclear localization sequences (NLSs; reviewed by Dingwall and Laskey, 1991). A different signal is responsible for nuclear import of small nuclear ribonucleoprotein particles containing certain U RNAs (U snRNPs) (Fischer et al., 1991; Michaud and Goldfarb, 1991), although its precise molecular nature is unknown. Furthermore, signals involved in RNA and protein export from the nucleus are just beginning to be characterized (Hamm and Mattaj, 1990; see also Schmidt-Zachmann et al., 1993).

Recent work has suggested that nuclear import can be regulated in several different ways (Nigg et al., 1991; Gerace, 1992). One type of mechanism affects the efficiency or availability of NLSs on individual proteins. For example this mechanism controls the nuclear import of certain transcription factors and protein kinases (Jans et al., 1991; Nigg et al., 1991; Moll et al., 1991; Henkel et al., 1992). A second type of mechanism appears to affect the protein import machinery rather than individual proteins (Feldherr and Akin,

1991). Clearly, regulated trafficking across the NPC can be important for control of gene expression and cellular function.

NLS-mediated nuclear protein import requires ATP and is inhibited at low temperatures (Newmeyer and Forbes, 1988; Richardson et al., 1988). An early step in nuclear import involves ligand "docking" at the cytoplasmic surface of the NPC, which can occur in the absence of ATP and at 0°C (Newmeyer and Forbes, 1988; Richardson et al., 1988). A subsequent step involves ligand translocation to the nucleoplasmic side of the NPC. The latter process involves channel gating (Feldherr et al., 1984), which is both ATP and temperature dependent. The precise components that mediate these and other steps of nuclear import are unknown.

Several *in vitro* systems that yield physiologically relevant mediated nuclear import have been developed recently (Newmeyer et al., 1986; Adam et al., 1990), providing valuable approaches for analyzing transport mechanisms. A system devised in our laboratory consists of digitonin-permeabilized cells (which retain an intact NE) supplemented with exogenous cytosol and ATP (Adam et al., 1990). Studies with this and other systems have demonstrated a requirement for multiple cytosolic factors in nuclear import (Adam et al., 1990; Newmeyer and Forbes, 1990; Moore and Blobel, 1992). One such factor is a protein that specifically binds NLSs with high affinity and has the properties of a functional import receptor (Adam et al., 1989; Adam and Gerace, 1991). Other cytosolic factors include a component that interacts with O-linked glycoproteins of the NPC (Sterne-Marr et al., 1992), the ubiquitous cellular protein hsc70 (Imamoto et al., 1992; Shi and Thomas, 1992), and several activities detected by fractionation of cytosol (Newmeyer and Forbes, 1990; Moore and Blobel, 1992).

It is now evident that GTPases are regulators of a vast diversity of cellular functions, including protein synthesis and translocation, signal transduction, and membrane transport (reviewed by Bourne et al., 1991; Pfeffer, 1992; Nuoffer and Balch, 1993). GTPases are molecular switches that exist in predominantly two conformations determined by the state of bound guanine nucleotide, an active form containing GTP and an inactive form containing GDP. The activity of GTPases is regulated by a combination of GTPase-activating proteins (GAPs), which stimulate the rate of GTP hydrolysis, and guanine nucleotide release proteins (GNRPs), which catalyze the release of bound GDP and allow GTPases to rebind GTP (Bourne et al., 1991).

We previously identified a small GTP-binding protein that is highly concentrated in the NE using photocross-linking approaches (Seydel and Gerace, 1991). In this study we have used an *in vitro* functional assay to directly investigate whether GTPases are involved in nuclear protein import. We found that nonhydrolyzable analogues of GTP, which often are potent inhibitors of GTPase function (Nuoffer and Balch, 1993), strongly inhibit nuclear protein import *in vitro*. We determined that the major component sensitive to nucleotide inhibition is Ran/TC4, a previously identified member of the Ras superfamily of GTP-binding proteins present in both nucleus and cytoplasm (Drivas et al., 1990; Bischoff and Ponstingl, 1991a; Ren et al., 1993). In addition, we found that Ran/TC4 is a cytosolic factor required for efficient nuclear import. Ran/TC4 and a protein called RCC1, which is a

GNRP for Ran/TC4 (Bischoff and Ponstingl, 1991b), have been previously implicated in a diverse range of nuclear functions, including DNA replication, cell cycle feedback control, and RNA processing and export (reviewed by Dasso, 1993). Our results suggest that Ran/TC4 serves to integrate nucleocytoplasmic trafficking with these other nuclear functions.

## Materials and Methods

### Cells and Reagents

Suspension cultures of human (HeLa) cells were grown in Joklik's-modified minimal essential medium (GIBCO-BRL, Gaithersburg, MD) with 10% FCS (Hyclone Laboratories, Logan, UT). Nucleotides and nucleotide analogs were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Nucleotide stock solutions (with equimolar Mg-acetate in 50 mM Hepes, pH 7.5) were aliquoted, stored in liquid nitrogen and used within 4 wk. Digitonin (high purity), creatine phosphate and creatine phosphokinase were obtained from Calbiochem-Behring Corp. (San Diego, CA). A 10% stock solution of digitonin in DMSO was stored in aliquots at -20°C. Stocks (10 mg/ml) of Avidin (Canadien Lysozyme, Abbotsford, Canada) and Biocytin (Sigma Immunochemicals, St. Louis, MO) were kept at 4°C. Purified ADP-ribosylation factor (ARF) from bovine brain was the generous gift of Dr. Frank Peter (The Scripps Research Institute, La Jolla, CA).

### Preparation of Transport Substrate

Synthetic peptides containing the SV-40 large T antigen wild-type NLS (CGGGPKKKRKVED) or a transport deficient "reverse" NLS peptide (CGGGDEVKRRKKK) were obtained from the Core Facility of TSRI and further purified by HPLC. Biotinylated BSA (B-BSA; Pierce Chemical Co., Rockford, IL) was activated with Sulfo-SMCC (Pierce Chemical Co.) and mixed with a 50-fold excess of peptide. After overnight incubation at 4°C the B-BSA-peptide conjugate was separated from free peptide using a PD10 column (Pharmacia Biotech Inc., Piscataway, NJ) and subsequent dialysis (25-kD molecular weight cut-off). The concentration of the conjugate was determined by absorbance at 280 nm. Aliquots were stored at -80°C. The number of peptides conjugated to the protein was estimated by mobility shift on SDS-polyacrylamide gels to be ~5-15 peptides per B-BSA molecule.

### Preparation of HeLa Cell Cytosol

Exponentially growing cultures of HeLa cells were collected by centrifugation at 250 g for 10 min and washed twice with ice cold PBS by resuspension and centrifugation. The cells were then washed with 10 mM Hepes, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, and 2 mM DTT. The cell pellet was resuspended in 1 vol of lysis buffer (5 mM Hepes, pH 7.3, 10 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 0.1 mM PMSF, and 1 µg/ml each of aprotinin, leupeptin, and pepstatin, and swelled for 10 min on ice. The cells were then lysed by five strokes in a tight fitting stainless steel dounce homogenizer. The resulting homogenates were centrifuged at 1,500 g for 15 min to remove nuclei and cell debris. The supernatant was then sequentially centrifuged at 15,000 g for 20 min and 100,000 g for 30 min. The final supernatant was dialyzed for 3-4 h with a collodion membrane apparatus (molecular weight cut-off of 10 kD; Schleicher & Schuell, Inc., Keene, NH) against transport buffer (20 mM Hepes pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 2 mM DTT, and 1 µg/ml of aprotinin, leupeptin, and pepstatin) with 0.1 mM PMSF and frozen in aliquots in liquid nitrogen before storage at -80°C. The protein concentration of the cytosol was 10-12 mg/ml as determined by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

### Preparation of ELISA Plates

Rabbit anti-BSA antibody preparations were obtained from Calbiochem-Behring Corp. and IgGs were purified by chromatography on protein G Sepharose. A solution of 4 µg/ml IgG in 50 mM Na<sub>2</sub>HCO<sub>3</sub>, pH 9.6 was plated onto immunomodule strips (Nunc Maxisorb F8 microwell immunomodules; Thomas Scientific, Swedesboro, NJ). Plates were incubated for 3 h at 37°C or overnight at 4°C, washed three times in PBS, and then incubated in blocking buffer (0.05% Tween 20 and 0.05% Gelatin in PBS)

for 1 h at 37°C. Plates were stored in blocking buffer at 4°C and used within 2 wk. Immediately before using, the strips were washed three times in PBS.

## Quantitative In Vitro Transport Assay

**Cell Permeabilization.** HeLa suspension cells were harvested by centrifugation, washed once in transport buffer and resuspended to a density of  $0.5 \times 10^7$  cells/ml. Digitonin was added to a final concentration of 0.008% and the cells were allowed to permeabilize for 7 min on ice. The suspension was diluted 10-fold with transport buffer, the cells were harvested and finally resuspended in the desired volume (normally  $2-8 \times 10^5$  cells/10  $\mu$ l).

**In Vitro Transport.** Transport mixtures (final volume 40  $\mu$ l) contained cytosol (usually 2-5 mg/ml), an ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, 20 U/ml creatine phosphokinase), transport substrate (100 nM biotinylated BSA-peptide), and permeabilized cells ( $2-8 \times 10^5$  cells) in transport buffer. The samples were mixed on ice in wells of Nunc microwell immunomodules, medium binding (Thomas Scientific). The transport reaction was started by transfer into a 30°C water bath and stopped, normally after 30 min, by dilution with 200  $\mu$ l of ice cold transport buffer and transfer of plates to ice. All experiments were carried out in triplicate to allow accurate quantitation.

**Wash and Lysis Conditions.** To remove and block remaining extranuclear transport substrate, the wells were spun for 5 min at 300 rpm in a Beckmann J6-B centrifuge, 200  $\mu$ l of the supernatant were removed and 100  $\mu$ l of avidin in transport buffer (0.1 mg/ml) was added to the cells. After 30 min at 4°C, 100  $\mu$ l biocytin in transport buffer (0.2 mg/ml) was added and the samples were allowed to sit for another 15 min. The samples were centrifuged again, 200  $\mu$ l supernatant were removed, and 200  $\mu$ l of lysis buffer (1.0% Triton X-100 in PBS) was added. The cells were thoroughly resuspended and allowed to lyse for at least 2 h or overnight at 4°C.

**ELISA-based Detection of Unblocked Transport Ligand.** 5  $\mu$ l of the cell lysate were added to 200  $\mu$ l of Triton buffer (0.2% Triton X-100 and 0.05% Gelatin in PBS) in immunomodule strips previously coated with rabbit anti-BSA antibodies. After incubation for 3 h at 37°C or overnight at 4°C, strips were washed three times with PBS, incubated for 5 min in Triton buffer and washed three times in PBS (this is referred to as a wash cycle). To each well 200  $\mu$ l of streptavidin-HRP (Boehringer-Mannheim Biochemical), diluted 1:5,000 in blocking buffer was then added. After 1 h at room temperature the strips were washed as before. 200  $\mu$ l substrate solution (10 mg *o*-phenylenediamine, 10  $\mu$ l H<sub>2</sub>O<sub>2</sub> in 25 ml of 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 27 mM citrate, pH 5) was added to each well and the incubation allowed to proceed until sufficient color was developed, typically 1-3 min. The reaction was terminated by addition of 50  $\mu$ l per well of 2 M H<sub>2</sub>SO<sub>4</sub>. The A<sub>490</sub> was read on an ELISA plate reader (Bio-Rad Laboratories) and corrected for the A<sub>655</sub>. Standard curves with transport substrate (0.1-4.0 ng per well) were carried out in parallel to allow for conversion of A<sub>490</sub> to ng of transported ligand.

## Test for Nuclear Integrity

Standard transport reactions were set up in either the presence or absence of 200  $\mu$ M GTP $\gamma$ S. FITC-Dextran (9,400 or 148,900 D, both from Sigma Immunochemicals) was added to a final concentration of 0.5 mg/ml 15 min after the start of the reaction. After another 15 min the cells were mounted onto coverslips and immediately photographed using a Zeiss Axiophot microscope and Kodak TMAX400 films. To visualize transport by fluorescence microscopy in this experiment, we used allophycocyanin coupled with NLS peptides as the transport ligand (Adam et al., 1990).

## Fractionation of Cytosol

Aliquots of cytosol were thawed and spun at 100,000 *g* for 10 min. The supernatant was then concentrated twofold using centricon-10 concentrators (Amicon Corp., Beverly, MA). 200  $\mu$ l of this cytosol were applied to a Superose 12 column connected to an FPLC system (Pharmacia) and 1-ml fractions were collected. Each fraction was concentrated to 100  $\mu$ l using again centricon-10 concentrators.

## Expression and Purification of Ran/TC4

A clone containing a cDNA for human Ran/TC4 was kindly provided by Dr. Peter D'Eustachio (New York University Medical School, New York). The coding region was amplified by PCR using primers that introduced two unique restriction sites at the termini, an NcoI site at the start codon and a BamHI site 5 bp after the stop codon. The fragment was ligated into the vector pET11d which was then transformed into *Escherichia coli* BL21-

(DE3). To obtain protein expression, transformed bacteria, freshly grown to an OD<sub>595</sub> = 0.6 were supplied with 1 mM IPTG and allowed to grow for 4 h at 37°C. The cells were harvested by centrifugation, resuspended in 10 mM Tris/HCl pH 8.0, 10% sucrose, 0.1 mM PMSF, 1  $\mu$ g/ml each of aprotinin, leupeptin, and pepstatin (5 ml buffer/50 ml culture) and frozen in liquid nitrogen. After thawing lysozyme was added to 1 mg/ml and the suspension was incubated for 40 min on ice, and then 1 mM MgCl<sub>2</sub> and 1 mM DTT were added. Soluble proteins obtained by centrifugation of cell lysates at 60,000 rpm in a Beckman TLA100.3 rotor for 40 min were applied to a DEAE-Sepharose column previously equilibrated in 10 mM Tris/HCl, pH 8.0, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM PMSF, 1  $\mu$ g/ml each of aprotinin, leupeptin, and pepstatin. The flow through and wash were discarded and Ran/TC4 was eluted with 100 mM NaCl. Fractions containing Ran/TC4 were pooled, concentrated, and applied onto a Superose 12 FPLC column equilibrated with transport buffer. Fractions containing Ran/TC4 were pooled and used either freshly or after storage at -80°C. The Ran/TC4 obtained by this procedure was ~80% pure. To account for effects of the contaminating proteins a control sample was obtained by carrying out the identical induction, lysis, and fractionation procedure with cells containing the vector pET11d only.

## Gel Electrophoresis and Immunoblotting

SDS-PAGE was carried out as described previously (Adam and Gerace, 1991). Detection of Ran/TC4 on immunoblots was performed as described (Ren et al., 1993) using antibodies kindly provided by Dr. Peter D'Eustachio.

## Results

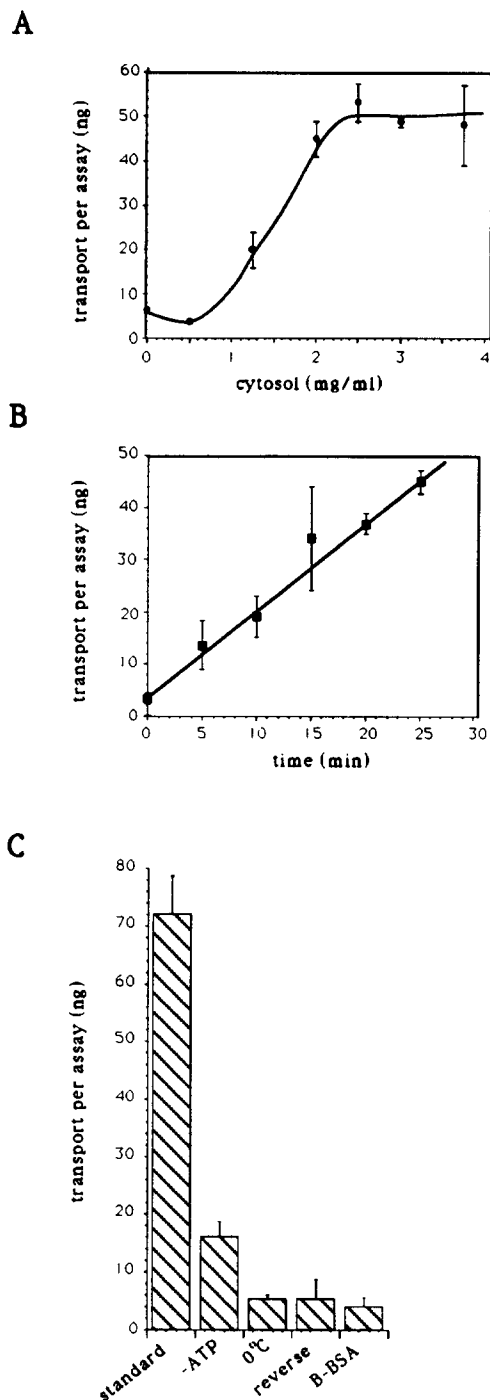
### An Improved Assay for Quantitative Analysis of In Vitro Nuclear Import

We adapted the in vitro assay for nuclear protein import developed by Adam et al. (1990) to include an ELISA-based detection system to allow rapid quantitative analysis of a large number of samples (see Materials and Methods). The transport ligand in this assay was B-BSA conjugated with synthetic NLS peptides. This new assay fulfills numerous criteria for physiologically relevant nuclear import. Transport depends on the presence of cytosol (Fig. 1 A), saturating at a cytosolic protein concentration of 2-3 mg/ml, and is dependent on temperature and ATP (Fig. 1 C). Furthermore, transport requires the presence of a wild-type NLS (CGG-GPKKKRKVED) on B-BSA, as B-BSA alone gave only background signal and B-BSA conjugated with a transport-deficient reverse NLS peptide (CGGGDEVKRRKKP; Adam et al., 1989) gave a very low signal (Fig. 1 C).

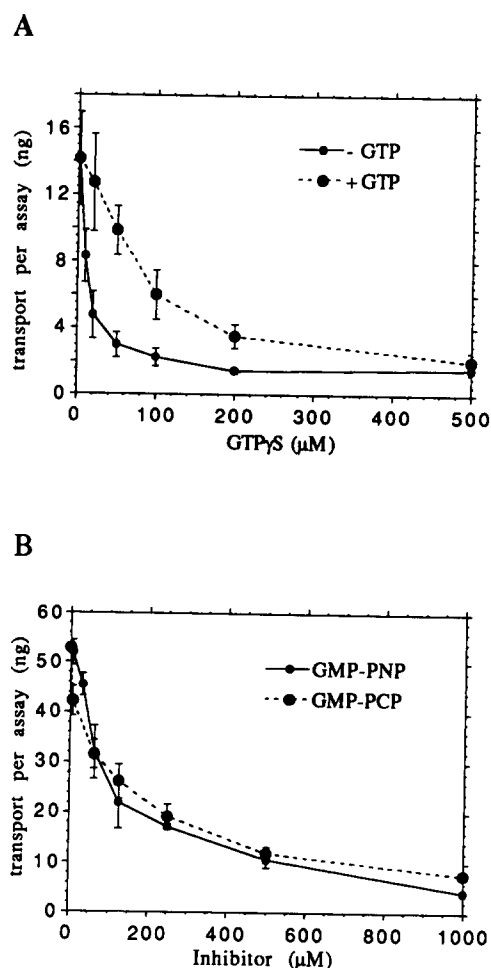
The accumulation of B-BSA-NLS in the nucleus is linear over at least 25 min. A representative experiment is shown in Fig. 1 B, where 45 ng of ligand were transported during 25 min in an assay containing  $6 \times 10^5$  cells. This is equivalent to roughly  $6.6 \times 10^5$  molecules per cell. Assuming there are  $2 \times 10^3$  active NPCs per nucleus (Gerace, 1992), this would reflect an average rate of about 1 molecule per NPC per 4 s. This in vitro transport rate obtained with our standard subsaturating concentration of ligand is in the same range as rates determined for in vivo nuclear import in *Xenopus* oocytes (Goldfarb et al., 1986).

### Nonhydrolyzable Analogues of GTP Specifically Inhibit Nuclear Protein Import

We found that addition of nonhydrolyzable GTP analogues to our in vitro nuclear import system resulted in virtually complete inhibition of transport under standard cytosol and cell concentrations (see below). The three analogues that we tested, GMP-PNP, GMP-PCP, and GTP $\gamma$ S were all effective



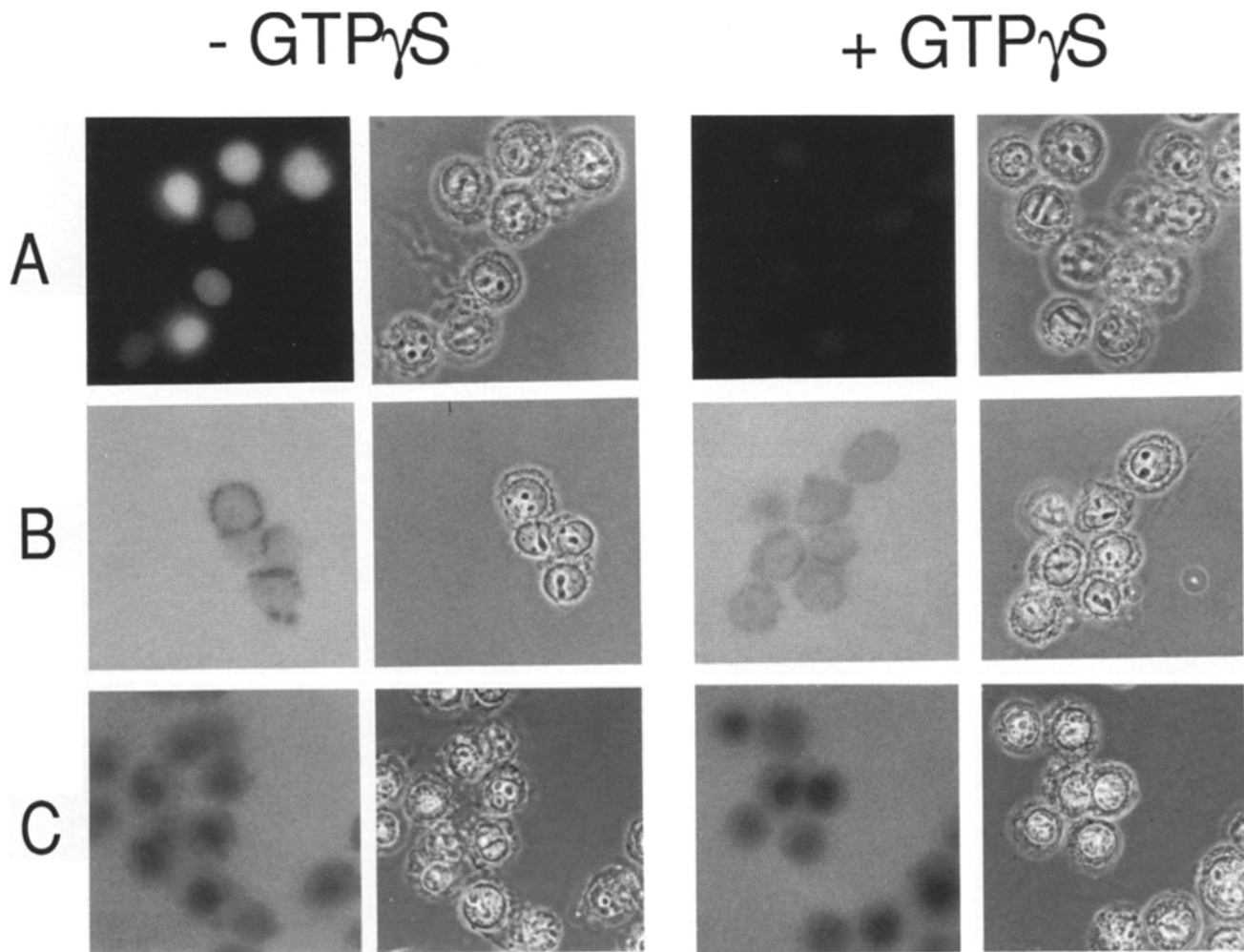
**Figure 1.** Characterization of an improved assay for quantitative analysis of in vitro nuclear import. Transport assays and measurement of transported ligand using an ELISA method were carried out as described in Materials and Methods. Each point represents the average of triplicate transport assays and error bars represent standard deviations. (A) Cytosol dependence of transport. The accumulation of biotinylated BSA-NLS (B-BSA-NLS) in the nucleus as a function of cytosol concentration in the reaction mix was determined after incubation of  $4 \times 10^5$  cells per assay for 30 min at 30°C. (B) Time course of transport. In vitro transport was carried out for the indicated times with  $6 \times 10^5$  cells per assay and 2.5 mg/ml cytosol. (C) Dependence of transport on ATP, temperature and nuclear localization sequence. All reactions were carried out for 30 min with 2.5 mg/ml cytosol and  $4 \times 10^5$  cells. (Standard) 30°C, B-BSA-NLS as transport ligand. (-ATP) addition of 0.1 U



**Figure 2.** Nonhydrolyzable analogues of GTP inhibit nuclear import. Transport was carried out in the presence of varying amounts of GTP analogues for 30 min with  $3 \times 10^5$  cells in the presence of an ATP regenerating system (see Materials and Methods). (A) Inhibition with GTPγS was measured in the presence or absence of 1 mM GTP in the transport mixture. The nucleotides were added to the complete transport mixture on ice immediately before the reaction was started. The final concentrations of GTPγS in the reaction are indicated. (B) The effect of varying amounts of the GTP analogues GMP-PNP and GMP-PCP in the assay were tested. The inhibitors were added to the complete transport mixture on ice immediately before the reaction was started. The difference in the total amount of protein transported in A vs. B is due to different ligand preparations used in the two assays.

in transport inhibition (Fig. 2). This inhibition was specific for guanine nucleotides, as it occurred in the presence of excess ATP (1 mM) but was diminished with an excess of GTP. As shown in Fig. 2 A, half-maximal inhibition was achieved with approximately 10 μM GTPγS in the absence of GTP or with 80 μM GTPγS in the presence of 1 mM GTP. With the other inhibitors, half maximal inhibition was achieved with somewhat higher concentrations, corresponding to ap-

hexokinase/5 mM glucose before start of the standard reaction. (0°C) 30 min at 0°C with standard ligand. (Reverse) B-BSA coupled to "reverse" NLS peptides as transport ligand under standard conditions. (B-BSA) Biotinylated BSA as transport ligand under standard conditions.



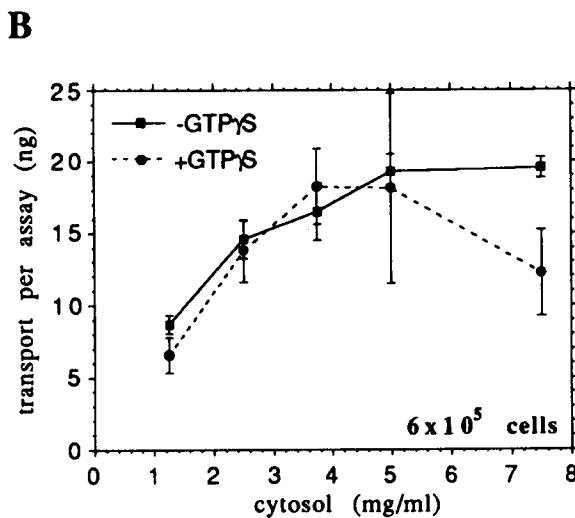
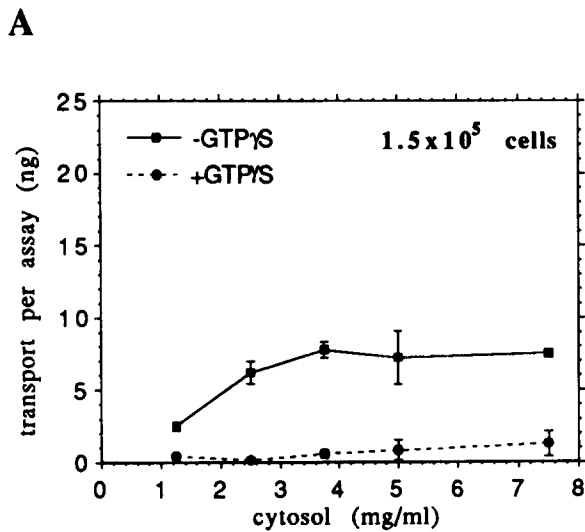
**Figure 3.** GTP $\gamma$ S does not affect nuclear integrity. Transport reactions were carried out in the presence or absence of 200  $\mu$ M GTP $\gamma$ S for 30 min using  $2 \times 10^5$  cells per assay and 2.5 mg/ml cytosol. The cells were then mounted onto coverslips without washing and photographed directly using epifluorescence (left micrograph of each pair) or phase contrast (right micrograph of each pair) optics. (A) Allopheycocyanin coupled to NLS-containing peptides was used as the transport ligand. (B) After 15 min of transport (using B-BSA-NLS as the transport ligand) 0.5 mg/ml fluorescein-dextran with a molecular weights of 9,400 D was added to the reaction. (C) After 15 min of transport (using B-BSA-NLS as the transport ligand) 0.5 mg/ml fluorescein-dextran with a molecular weight of 149,000 D was added to the reaction.

proximately 100  $\mu$ M GMP-PNP or 100  $\mu$ M GMP-PCP (Fig. 2 B). The observation that all three analogues inhibited transport argues against the possibility that thiophosphorylation of ATP led to transport inhibition. Instead these data suggest that hydrolysis of GTP is involved in nuclear protein import.

The inhibitory effect of GTP $\gamma$ S on nuclear import was also evident when a fluorescent ligand (allopheycocyanin coupled with synthetic NLS peptides; Adam et al., 1990) was used to monitor transport. Addition of 200  $\mu$ M GTP $\gamma$ S to the assay greatly reduced nuclear ligand accumulation compared to a control sample (Fig 3 A). Interestingly, in the presence of nonhydrolyzable GTP analogues we never observed accumulation of fluorescent ligand in a rimlike pattern at the nuclear periphery, which is indicative of ligands docked at the NPC (Newmeyer and Forbes, 1988; Richardson et al., 1988). If GTP hydrolysis were required only at a transport step distal to docking, one would expect accumulation of ligand in a docked configuration in the presence of GTP $\gamma$ S.

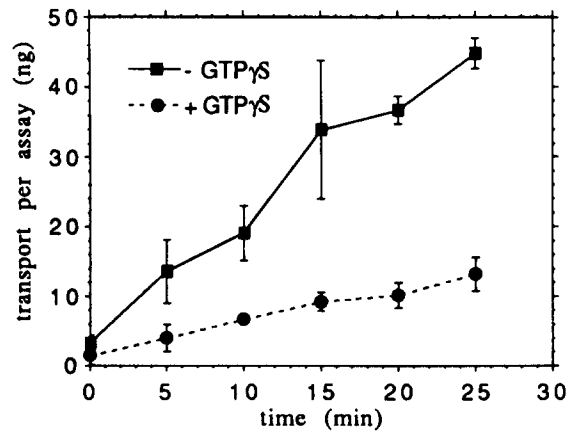
In other experiments, we investigated the effects of GTP $\gamma$ S on docking by carrying out nuclear import assays at 30°C under conditions of ATP depletion (Newmeyer and Forbes, 1988; Richardson et al., 1988), wherein docked ligands accumulate at the cytoplasmic surface of the NPC (Newmeyer and Forbes, 1988; Richardson et al., 1988). In this case, we found that GTP $\gamma$ S strongly inhibited the rate of accumulation of ligands at the nuclear periphery (data not shown), supporting the notion that GTP hydrolysis is required at a transport step prior to or involving ligand docking at the NPC.

To test whether the effects of GTP $\gamma$ S are trivially due to disruption of nuclear integrity or wholesale occlusion of NPCs, we carried out transport in the presence of GTP $\gamma$ S and one of two different fluorescent dextrans (Figs. 3, B and C). An  $\sim$ 10-kD dextran, which is small enough to readily diffuse into the nucleus *in vivo*, was used to measure diffusional permeability of the NPC, and an  $\sim$ 150-kD dextran, which is too large to diffuse into an intact nucleus in



**Figure 4.** Inhibition by GTP $\gamma$ S is dependent on concentrations of permeabilized cells and cytosol. The effect of 200  $\mu$ M GTP $\gamma$ S on in vitro transport was measured as a function of cytosol concentration for  $1.5 \times 10^5$  cells (A) or for  $6 \times 10^5$  cells (B) per 40- $\mu$ l transport reaction. Transport and determination of the amount of ligand transported per assay were otherwise carried out as described in Materials and Methods. As the degree of inhibition obtained by GTP $\gamma$ S varied with the batch of cytosol and also with the preparation of digitonin permeabilized cells, the experiments in this figure were done in parallel using the same materials for A and B.

vivo, was used to measure nuclear intactness (Paine et al., 1975; Peters, 1986). As shown in Fig. 3 B, the low molecular weight dextran accumulated in nuclei of the GTP $\gamma$ S-treated sample similar to the control sample, indicating that the diffusional channel of the NPC is not detectably occluded. The 150-kD dextran was excluded from nuclei whether or not GTP $\gamma$ S was present (Fig. 3 C), indicating that the nuclear envelope does not become disrupted by the nucleotide analogue. A separate approach also led to the conclusion that the inhibitor did not compromise nuclear integrity: transport was initially carried out in the absence of inhibitor and at different time points up to 30 min 100  $\mu$ M GTP $\gamma$ S was added. The transport reaction was then continued for the

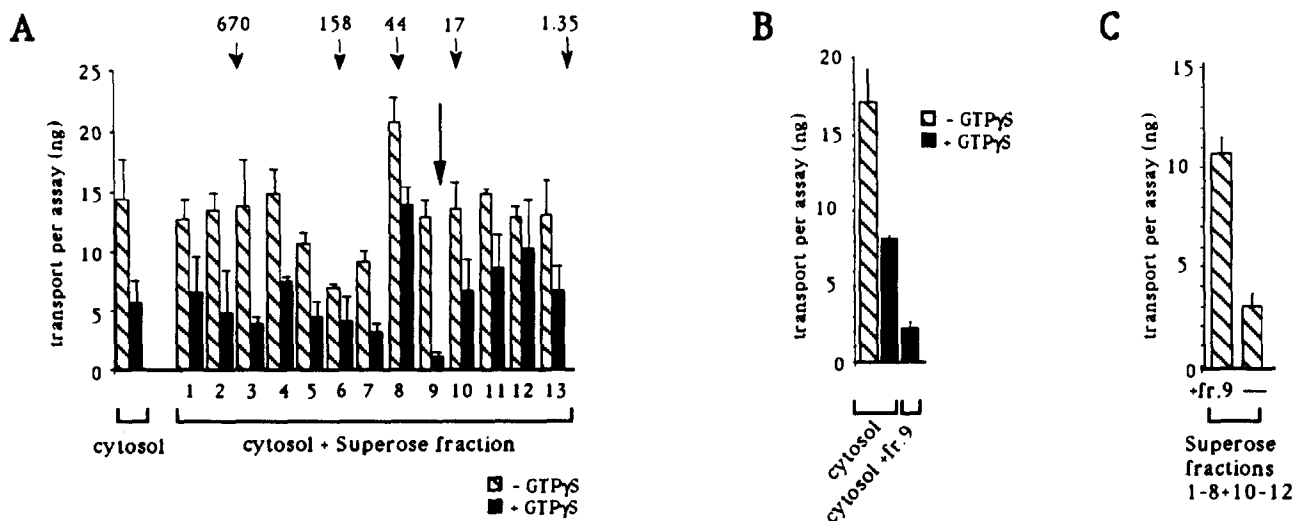


**Figure 5.** GTP $\gamma$ S affects the rate of nuclear protein import. Accumulation of ligand over time was measured in the presence or absence of 200  $\mu$ M GTP $\gamma$ S. 2.5 mg/ml cytosol and  $6 \times 10^5$  cells were used per assay. The transport was stopped at the indicated times and processed as described in Materials and Methods. In the experiment shown,  $6 \times 10^5$  cells led to 70% inhibition, while in a similar experiment conducted with a different batch of cytosol the same degree of inhibition and the same shape of the curve was achieved with  $3 \times 10^5$  cells (data not shown).

remainder of the 30 min, before the samples were processed to measure nuclear accumulation of ligand. This experiment showed that ligand which had accumulated in the nuclei at the time of GTP $\gamma$ S addition remained in the nucleus during the subsequent incubation with GTP $\gamma$ S (data not shown). We conclude from these data that GTP $\gamma$ S does not affect nuclear integrity nor does it detectably block the diffusional channel of the NPC.

#### Transport Inhibition Is Dependent on Concentrations of Cells and Cytosol

The inhibitory effects of GTP $\gamma$ S on nuclear import were strongly influenced by both cell and cytosol concentrations in the assay (Fig. 4). While low cell concentrations yielded almost complete transport inhibition with GTP $\gamma$ S, higher cell concentrations resulted in markedly reduced or no transport inhibition. As shown in Fig. 4 A, when  $1.5 \times 10^5$  cells per 40- $\mu$ l reaction was analyzed, GTP $\gamma$ S yielded virtually complete transport inhibition over a broad range of cytosol concentrations. By contrast, essentially no inhibition was obtained with  $6 \times 10^5$  cells per 40- $\mu$ l reaction up to a cytosol concentration of 5 mg/ml. However, further increasing the cytosol concentration in this sample again resulted in transport inhibition. An intermediate concentration of cells in the assay ( $3 \times 10^5$  cell per 40- $\mu$ l reaction) yielded a level of inhibition intermediate between that obtained with  $1.5 \times 10^5$  cells and  $6 \times 10^5$  cells (data not shown; see also Fig. 5). Hence, depending on the concentration of cytosol and cells in the assay, the inhibition can vary from 0 to 95%. This phenomenon was observed with all cytosol and permeabilized cell preparations tested, although it should be emphasized that the concentration of cells required to obtain specific degrees of transport inhibition varied with different cytosols as well as with different permeabilized cell preparations. We presently have no clear understanding of this effect, which could involve complex interactions between nuclear and cytoplasmic pools of GTP-binding proteins and



**Figure 6.** A low molecular weight fraction is required for nuclear protein import and enhances the inhibitory effects of GTP $\gamma$ S. (A) A Superose 12 column was used to fractionate cytosol into 13 fractions. Each fraction was then concentrated fourfold (with respect to the initial cytosol concentration) and tested for its effects when added to a standard transport assay in the presence or absence of 200  $\mu$ M GTP $\gamma$ S. The standard transport assay contained 2.5 mg/ml complete cytosol and  $3 \times 10^5$  cells, and the amount of each added fraction was equivalent to that derived from 40  $\mu$ l complete cytosol. (Cytosol) Standard assay without additions; (1-13) transport with the standard mixture plus each of the individual concentrated cytosol fractions. The small arrowheads indicate the position of molecular weight standards in the Superose column profile. (B) An independent experiment with a different batch of cytosol was carried out as described above in A: cytosol or cytosol enriched with fraction 9 (equivalent to 40  $\mu$ l complete cytosol) was tested in the absence or presence of 200  $\mu$ M GTP $\gamma$ S. (C) An experiment was carried out with assay samples containing permeabilized cells without complete cytosol but containing the combined Superose fractions 1-12 (+fr.9) or combined fractions 1-8 + 10-12 (-) only. (▨) Transport in the absence of GTP $\gamma$ S; (■) transport in the presence of 200  $\mu$ M GTP $\gamma$ S.

their regulators. Nevertheless, this observation was crucial for identifying the transport factor sensitive to GTP $\gamma$ S (see below).

Fig. 5 shows the kinetics of ligand accumulation in the nucleus in the presence or absence of GTP $\gamma$ S under conditions which lead to 70% inhibition at 30 min. The transport rate was linear throughout the time course both in the presence or absence of GTP $\gamma$ S, but the rate of transport was strongly reduced in the presence of inhibitor. It is important to note that 70% inhibition was already seen during the first 5 min of the reaction, so there is no apparent lag in the effects of the inhibitor. Hence, GTP $\gamma$ S continuously affects the rate of transport throughout the time course of this assay in a cytosol and permeabilized cell concentration-dependent fashion.

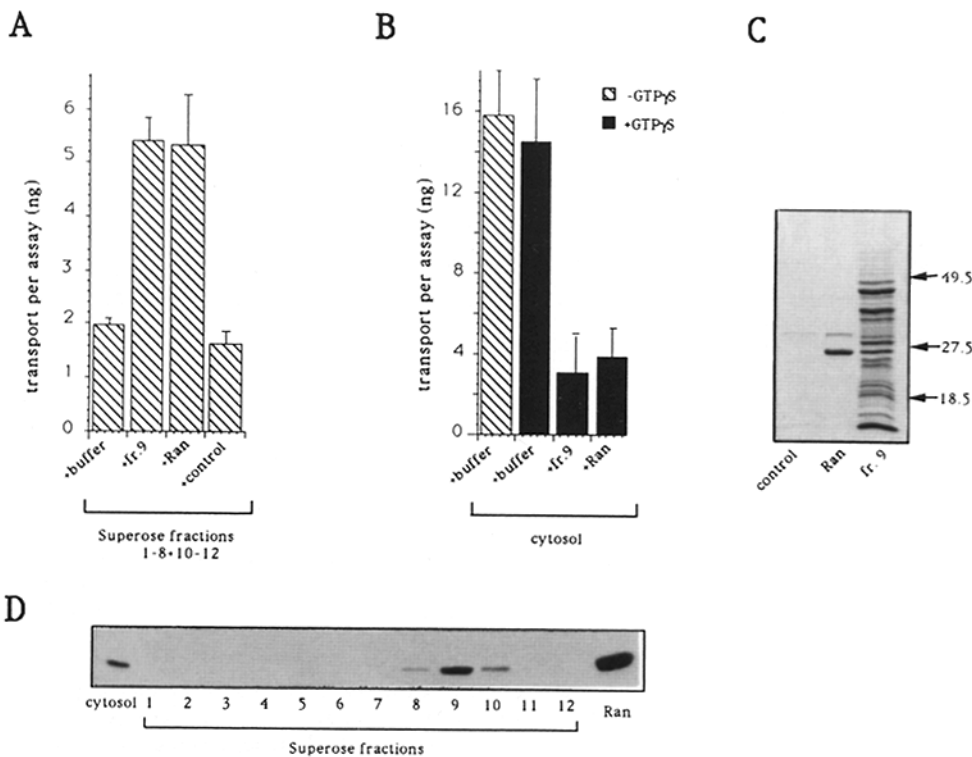
#### ***A Low Molecular Weight Cytosolic Fraction Is Responsible for GTP $\gamma$ S-Mediated Inhibition and Is Required for Transport***

As an approach to characterizing the transport factor inhibited by GTP $\gamma$ S, we examined whether inhibition could be stably maintained after a brief exposure of transport components to the GTP analogue. We preincubated either permeabilized cells or cytosol or a combination of both with GTP $\gamma$ S at 30°C, added an excess of GTP to compete with the inhibitor (see Fig. 2 A), and then compared transport in the pretreated fraction with untreated counterparts. However, no irreversible inhibition was achieved under any conditions tested (data not shown), indicating that GTP $\gamma$ S must be present in the assay throughout the transport incubation to exert inhibitory effects. We therefore explored a second approach for characterizing the GTP $\gamma$ S sensitive factor.

Since a high concentration of cytosol can substantially in-

crease the level of transport inhibition by GTP $\gamma$ S in an assay containing a relatively high cell concentration (Fig. 4 B), we reasoned that cytosol contains a factor responsible for mediating or enhancing GTP $\gamma$ S inhibition in a concentration-dependent manner. To identify this factor, we resolved cytosol into 13 fractions by molecular sieving chromatography, concentrated each fraction fourfold (relative to the initial cytosol) and individually added the fractions to transport mixtures containing complete cytosol in the absence or presence of GTP $\gamma$ S (Fig. 6). The concentration of permeabilized cells in the transport incubation was adjusted to yield about 50% transport inhibition in the presence of GTP $\gamma$ S without added cytosolic fractions (Fig. 6 A, leftmost bars). Addition of most cytosol fractions to the transport assay had no significant effect, either on the transport rate without GTP $\gamma$ S or on the degree of inhibition obtained with GTP $\gamma$ S. A few fractions enhanced (e.g., fraction 8) or diminished (e.g., fraction 6) the basal level of transport in the absence of GTP $\gamma$ S, but the relative degree of inhibition in the presence of GTP $\gamma$ S was similar to the control. A strikingly different result was obtained with fraction 9, which corresponds to the peak of 20-30-kD globular proteins (arrow in Fig. 6). Addition of concentrated fraction 9 did not significantly influence the level of transport in the absence of inhibitor, yet dramatically increased the degree of transport inhibition obtained with GTP $\gamma$ S to >90%. The ability of fraction 9 to strongly enhance the inhibitory effect of GTP $\gamma$ S on transport was highly reproducible. For example, Fig. 6 B presents a separate experiment with a different batch of fractionated cytosol. To determine whether this low molecular weight fraction was also required for transport, we pooled fractions 1-8 + 10-12 and tested the transport activity of this





**Figure 7.** Bacterially expressed Ran/TC4 is the major component of the low molecular weight fraction required for nuclear import and inhibition by GTP $\gamma$ S. Ran/TC4 was expressed in *E. coli* as described in Materials and Methods and was enriched by ion exchange chromatography and molecular sieving on a Superose 12 column. To control for impurities, a mock preparation was done in parallel from *E. coli* containing the expression vector only without the Ran/TC4 insert. (A) Superose fractions of cytosol were obtained as described in Fig. 6. Fractions 1–8 + 10–12 were tested either alone (*buffer*) or supplemented with 10- $\mu$ l fraction 9 (+fr.9), 7  $\mu$ l purified Ran/TC4 (*Ran*) or 7  $\mu$ l mock-purified Ran/TC4 (*control*), using  $3 \times 10^5$  cells per assay. (B) Unfractionated cytosol was tested alone (*buffer*) or

supplemented with 10- $\mu$ l fraction 9 (+fr.9) or 10  $\mu$ l purified Ran/TC4 (*Ran*) in the presence or absence of 200  $\mu$ M GTP $\gamma$ S, using  $6 \times 10^5$  cells per assay. (C) SDS-PAGE of the mock preparation isolated from *E. coli* (*control*), the Ran/TC4 preparation (*Ran*), and fraction 9 (*fr.9*). 5  $\mu$ l of each sample was applied to the gel, which was stained with Coomassie blue. (D) Immunoblot analysis of cytosol fractions obtained by molecular sieving on Superose 12 using antibodies against Ran/TC4. (Cytosol) Complete cytosol; (1–12) 7  $\mu$ l of fractions 1–12 respectively (threefold concentrated over cytosol); (*Ran*) 7  $\mu$ l of the bacterially expressed Ran/TC4.

pool alone or with added fraction 9 (Fig. 6 C). When fraction 9 was omitted, the transport activity was greatly diminished, demonstrating that some factor(s) in this fraction is required for efficient nuclear import.

### The Small GTPase Ran/TC4 Is a GTP $\gamma$ S-Sensitive Transport Factor Involved in Nuclear Protein Import

The data above provided evidence for a 20–30-kD cytosolic protein which was required for nuclear protein import and which enhanced inhibitory effects of GTP $\gamma$ S. It seemed plausible that this component itself was a GTPase, since GTPases are of widespread importance as cellular regulators and are often strongly inhibited by GTP $\gamma$ S. Furthermore, many GTPases occur in this molecular weight range, most notably, GTPases of the Ras superfamily (Bourne et al., 1991). Two small GTPases have been previously suggested to have potential roles in nuclear function, the 25-kD protein Ran/TC4 and the 20-kD protein ARF (ADP ribosylation factor). Ran/TC4 appears to be directly or indirectly involved in a large number of nuclear functions including DNA replication, cell cycle feedback control, and RNA synthesis, processing and export (Dasso, 1993). ARF has been shown to be responsible for inhibition of *in vitro* nuclear reassembly in the presence of GTP $\gamma$ S (Boman et al., 1992), and also has an extensively characterized role in vesicle budding along the secretory pathway (Pfeffer, 1992; Nuoffer and Balch, 1993).

We therefore examined whether either of these two proteins was the component of fraction 9 required for nuclear import. Purified ARF from bovine brain, which was active in an ER to Golgi membrane transport assay (Nuoffer and Balch, 1993), had no effect when added to an assay containing a pool of all cytosol fractions except fraction 9 (data not shown). By contrast, striking results were obtained with purified recombinant Ran/TC4. Addition of Ran/TC4 to the transport assay lacking fraction 9 increased the level of nuclear transport in this sample to the degree obtained with concentrated fraction 9 itself (Fig. 7 A; see also Fig. 6 C). This effect was not due to bacterial impurities in the Ran/TC4 preparation, since a control sample purified from control bacterial lysates and containing all of the minor contaminant bands found in the Ran/TC4 sample (Fig. 7 C, compare *Ran* lane to *control* lane) was without effect. We estimate that the amount of recombinant Ran/TC4 added to the transport assays in Fig. 7 is no more than severalfold greater than the amount of endogenous cytosolic Ran based on the relative immunoblot signals given by the two samples (Fig. 7 D). Saturating concentrations of Ran/TC4 were added to the samples in Fig. 7 since up to fourfold lower concentrations of added Ran yielded the same effect (data not shown). This suggests a direct involvement of Ran/TC4 in nuclear import. Supporting this conclusion, the chromatographic peak of cytosolic Ran/TC4 coincides with fraction 9, as determined by immunoblotting with antibodies to Ran/TC4 (Fig. 7 D). The residual level of transport in cytosol lacking fraction 9



is most likely due to the small amount of Ran/TC4 in fractions 8 and 10 which is not resolved by molecular sieving, although we cannot formally exclude the possibility that a low level of nuclear import can occur in the absence of cytosolic Ran/TC4.

In a second experiment, we examined whether Ran/TC4 was responsible for inhibition by GTP $\gamma$ S in this assay (Fig. 7 B). Recombinant Ran/TC4 was added to a transport assay that was adjusted to give a low level of inhibition by GTP $\gamma$ S in the absence of additions. We found that addition of exogenous Ran/TC4 to the system yielded virtually the same degree of transport inhibition as concentrated fraction 9 itself. In conclusion, these data directly demonstrate that Ran/TC4 is a cytosolic factor involved in nuclear import, and that this protein is a major factor involved in inhibition of transport by GTP $\gamma$ S.

## Discussion

We have shown that GTP $\gamma$ S and other nonhydrolyzable GTP analogues inhibit the rate of nuclear import in permeabilized cells. We identified the GTP $\gamma$ S-sensitive component as Ran/TC4 and demonstrated that this protein is required for efficient nuclear protein import. It is possible that additional GTPases besides Ran/TC4 are involved in nuclear import, although these are not clearly revealed with our standard assay and inhibition conditions. A requirement for Ran/TC4 in nuclear protein import also was demonstrated with an *in vitro* system involving *Xenopus* cytosol, indicating a role in diverse cell types (Moore and Blobel, 1993).

Ran/TC4 was first identified in mammalian cells as a member of the Ras superfamily of GTPases having about 25% identity to Ras (Drivas et al., 1990; Bischoff and Ponstingl, 1991a). It also was genetically defined as a multicopy suppressor of mutations in yeast *RCC1* homologues (see below; Matsumoto and Beach, 1991; Belhumeur et al., 1993; Kadowaki et al., 1993). Ran/TC4 is concentrated in the nucleus of mammalian cells based on immunofluorescence microscopy, but a smaller cytoplasmic pool is also present and is released by permeabilization of cells with digitonin (Ren et al., 1993). The relatively high concentration of Ran/TC4 in the nucleus is presumably due to its interaction with other nuclear components, since monomeric Ran/TC4 is small enough to rapidly diffuse into the cytoplasm. Nevertheless nuclear Ran/TC4 may be in dynamic equilibrium with the cytoplasmic pool, and this equilibrium could be important for its functions. The subcellular distribution of Ran/TC4 could help to explain the variable inhibitory effects of GTP $\gamma$ S related to the concentrations of permeabilized cells and cytosol in the transport assay (Fig. 4) assuming the nuclear and cytoplasmic pools are differentially sensitive to GTP $\gamma$ S. Since there are an estimated  $10^7$  copies of Ran/TC4 per mammalian cell (Bischoff and Ponstingl, 1991a), recycling of this protein would not be required to drive the levels of transport obtained in our assay conditions ( $<10^6$  molecules per cell), even if Ran/TC4 is a stoichiometric component of the transport apparatus (see below).

Our results on the role of Ran/TC4 in nuclear import are intriguing in light of recent work on Ran/TC4 and the protein RCC1, which is a GNRP for Ran/TC4 (Bischoff and Ponstingl, 1991b). RCC1 was first characterized in the hamster cell line tsBN2 (Nishimoto et al., 1978), which contains a

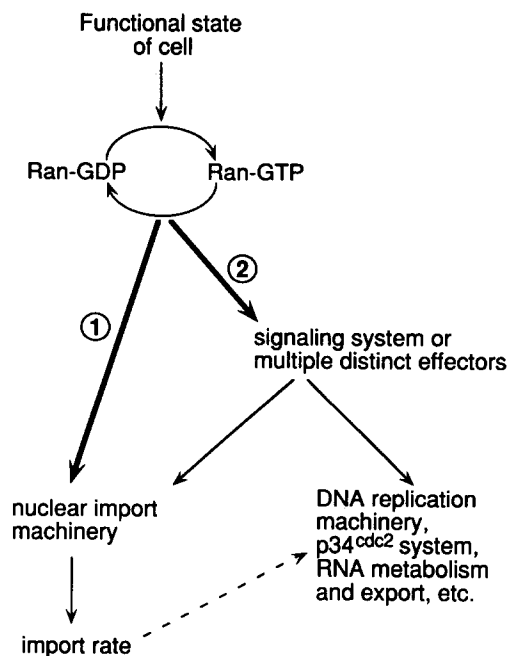


Figure 8. Two possible pathways by which Ran/TC4 can integrate nuclear functions. See text for discussion.

mutant RCC1 protein that is rapidly degraded at the restrictive temperature (Nishitani et al., 1991). TsBN2 cells that are in S phase at the time of the temperature shift enter mitosis without completing DNA replication (Nishimoto et al., 1978), leading to the view that RCC1, which is a chromatin bound nuclear protein, is part of the checkpoint control mechanism for preventing entry into mitosis until completion of DNA replication (reviewed by Dasso, 1993). A homologue of RCC1 with a similar mutant phenotype (*pim1*) was identified in a genetic screen in *Schizosaccharomyces pombe* (Matsumoto and Beach, 1991). RCC1 has been linked to a large number of additional functions, based on genetic and biochemical studies of RCC1 homologues in *S. cerevisiae* and vertebrate cells (Aebi et al., 1990; Clark et al., 1991; Dasso et al., 1992; Forrester et al., 1992; Kadowaki et al., 1993; Amberg et al., 1993). These include a role in RNA synthesis, processing, and export as well as in DNA replication. Recent functional studies indicate that mutations in Ran/TC4 can result in a deficiency in DNA replication (Ren et al., 1993), similar to RCC1 mutants. Hence, the Ran/TC4-RCC1 system is directly or indirectly associated with a large number of processes underlying nuclear function.

The results on Ran/TC4 described in this study in combination with previous work indicate that the Ran/TC4-RCC1 system can integrate numerous nuclear activities (Fig. 8). The functional state of the cell (related to DNA replication and possibly other processes) is thought to influence the GNRP activity of RCC1 and perhaps other GNRPs (Dasso, 1993), and probably affects the activity of hypothetical Ran/TC4-GAPs as well. This combination of GNRP and GAP activities would determine the relative amounts of Ran/TC4-GTP and Ran/TC4-GDP in the cell, and thereby the activity of the Ran/TC4 system. We assume that in analogy with other GTPases (Bourne et al., 1991) the active form

of Ran/TC4 is GTP-bound. According to one scenario (pathway 1 in Fig. 8), active Ran/TC4 could interact with only a single target in the cell, which would be associated with the nuclear import machinery. As such, Ran/TC4 could serve as a stoichiometric regulator of nuclear import (similar to the role of EF-Tu in protein synthesis; Bourne et al., 1991). When the Ran/TC4 system is relatively inactive, the rate of nuclear import would decrease. This could indirectly lead to inhibition of DNA replication and defects in cell cycle checkpoint control due to inappropriate concentrations of regulatory molecules in the nucleus compared to the cytoplasm.

In a second scenario (pathway 2 in Fig. 8) active Ran/TC4 could interact with multiple targets in the cell either through a signaling system (e.g. a protein kinase) or through multiple distinct effectors. In this fashion Ran/TC4 could coordinately regulate both the nuclear import machinery and other functional components, such as the DNA replication machinery and the p34<sup>cdc2</sup> kinase system.

The observed effects of the Ran/TC4-RCC1 on RNA export could be direct, and Ran/TC4 could affect the nuclear export machinery in a fashion similar to the import machinery. This is compatible with the notion that at least some steps of both nuclear import and export (e.g., channel gating) are likely to be mechanistically similar (Gerace, 1992). Alternatively, the effects of Ran/TC4-RCC1 on RNA export could be a consequence of deficiencies in nuclear import. This possibility is consistent with the observation that numerous RNA binding proteins continuously "shuttle" between nucleus and cytoplasm (Borer et al., 1989; Meier and Blobel, 1992; Pinol-Roma and Dreyfuss, 1992). Trapping these shuttling proteins in the cytoplasm due to deficient nuclear import could have profound effects on nuclear RNA metabolism and export.

By analogy with other GTPases (Bourne et al., 1991; Nuoffer and Balch, 1993), we predict that the active form of Ran/TC4 affecting nuclear import is the GTP-bound form. The inhibitory effects of GTP $\gamma$ S in our nuclear import assay are compatible with either pathway 1 or pathway 2 in Fig. 8, assuming that the functions of Ran/TC4 require hydrolysis of bound GTP. If Ran/TC4 is involved in nuclear import according to pathway 1, GTP hydrolysis most likely would occur at a specific step in transport. Conversely, if Ran/TC4 functions according to pathway 2, then multiple components and steps involved in nuclear import could be affected. We speculate that a transport step prior to or involving ligand docking at the NPC requires GTP hydrolysis, since ligands do not accumulate in a docked configuration at the NE when transport is inhibited by GTP $\gamma$ S (this study) or GMP-PNP (Moore and Blobel, 1993). Furthermore, the rate of accumulation of fluorescent transport ligands at the NE is strongly diminished by GTP $\gamma$ S under standard docking conditions of ATP depletion. Whether the GTP $\gamma$ S sensitive step proximal to docking involves Ran/TC4 or another unidentified GTPase remains to be established.

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