The exchange of molecular information between the nucleus and cytoplasm is mediated via a bidirectional trafficking pathway through the nuclear pore complexes (NPC) in the nuclear envelope (reviewed by Forbes, 1992; Rout and Wente, 1994). The selective import of karyophilic proteins through the pore into the nucleus is a multistep process (recently reviewed by Moore and Blobel, 1994a; Melchior and Gerace, 1995; Simos and Hurt, 1995). In vitro assays that support nuclear protein import in permeabilized cells have allowed the identification of soluble protein fractions that reconstitute these steps (Newmeyer and Forbes, 1990; Adam et al., 1990; Moore and Blobel, 1992). In the first step, a protein bearing a nuclear localization signal (NLS) (reviewed by Dingwall and Laskey, 1991; Garcia-Bustos et al., 1991) is targeted to the NPC. This step is mediated by an NLS receptor, a heterodimer of α- and β-karyopherin (Adam and Adam, 1994; Görlich et al., 1994, 1995; Weis et al., 1995; Radu et al., 1995; Moroianu et al., 1995a,b; Imamoto et al., 1995a,b). In subsequent steps, which require the abundant GTP/GDP-binding protein Ran and GTP hydrolysis, the NLS-bearing protein is transported from the initial site of docking to the central gated channel of the NPC and into the nucleus (Moore and Blobel, 1993; Melchior et al., 1993, 1995; Schlenstedt et al., 1995). Hydrolysis of Ran-GTP is stimulated by the RanGAP Rna1, which is also required for nuclear protein import in vivo and in vitro (Corbett et al., 1995; Bischoff et al., 1994, 1995a,b). This step also requires a second protein called p10/NTF2 (Moore and Blobel, 1994b; Paschal and Gerace, 1995). NTF2 has been shown to bind to the NPC protein p62 localized in the direct vicinity of the gated channel, but its biochemical activity is unknown. Ran, NTF2, and α-karyopherin are all transported into the nucleus, presumably with the transport substrate, while β-karyopherin remains bound at the NPC (Moroianu et al., 1995a,b; Görlich et al., 1995).

RCC1 (regulator of chromosome condensation 1; reviewed by Dasso, 1993) was first identified as the mutant gene responsible for the temperature-sensitive phenotype of the cell line tsBN2 (Nishimoto et al., 1978). RCC1 is involved directly or indirectly in the control of cell cycle progression, nuclear structure, DNA replication, RNA transcription, processing, and export (Dasso, 1993). The phenotypic defects caused by some conditional mutations in RCC1 homologs in yeast can be suppressed by overexpression of Ran homologs in vivo.
(Matsumoto and Beach, 1991; Belhumeur et al., 1993; Kadowaki et al., 1993; Sazer and Nurse, 1994). RCC1 has been shown to catalyze guanine nucleotide exchange on Ran in vitro (Bischoff and Ponstingl, 1991a,b). The guanine nucleotide exchange activity of the mutant RCC1 was lost 20 minutes after tsBN2 extracts were shifted to the nonpermissive temperature (Bischoff et al., 1995a). RCC1 was not detected by immunofluorescence in tsBN2 cells at the nonpermissive temperature, leading to the notion that RCC1 undergoes degradation (Nishitani et al., 1991; Ren et al., 1993; Tachibana et al., 1994).

The role of Ran in nuclear protein import and the genetic and biochemical interactions between Ran and RCC1 raised the question of whether RCC1 might also play a role in nuclear protein import. Initial studies of nuclear protein import in tsBN2 cells at the nonpermissive temperature detected no defect (Kadowaki et al., 1993), but while this manuscript was in preparation, nuclear protein import was reported to be reduced at the nonpermissive temperature in tsBN2 cells that were starved for serum and isoleucine (Tachibana et al., 1994). In the present report, we present quantitative evidence to confirm the role of RCC1 in nuclear protein import in vivo. We also demonstrate that the temperature-sensitive defect in tsBN2 cells can be suppressed by supplying additional Ran in the cytoplasm, while Ran mutants trapped in either the GTP- or GDP-bound form do not relieve the defect.

MATERIALS AND METHODS

Preparation of microinjected proteins and snRNPs

U1snRNPs were purified and labelled as described (Marshallsay and Basilico, 1978), a temperature-sensitive mutant derived from BHK21 (Stoker and MacPherson, 1978), a temperature-sensitive mutant derived from BHK21 (Stoker and MacPherson, 1964) and tsBN2 cells (Nishimoto and Basilico, 1978), were grown in Dulbecco’s modified Eagle medium (ccpro GmbH, Karlsruhe, Germany) supplemented with antibiotics and 10% fetal calf serum (FCS) (BioChrom, Berlin, Germany) in a humidified incubator at 37°C (BHK21) or 33.5°C (tsBN2, permissive temperature) under a 10% CO2 atmosphere.

Cell culture

BHK21 (Stoker and MacPherson, 1964) and tsBN2 cells (Nishimoto and Basilico, 1978), a temperature-sensitive mutant derived from BHK21, were grown in Dulbecco’s modified Eagle medium (ccpro GmbH, Karlsruhe, Germany) supplemented with antibiotics and 10% fetal calf serum (FCS) (BioChrom, Berlin, Germany) in a humidified incubator at 37°C (BHK21) or 33.5°C (tsBN2, permissive temperature) under a 10% CO2 atmosphere.

Microinjection

For microinjection experiments, cells were plated at least 36 hours before microinjection on Cellocate glass coverslips (Eppendorf, Hamburg, Germany). A microinjector and a manipulator (Eppendorf models 3242 and 5170) mounted on an IM35 inverted microscope (Carl Zeiss, Oberkochen, Germany) were used to deliver samples. All proteins used for injection were mixed as indicated in the figure legends, centrifuged for 15 minutes at 14,000 g, and injected at the final concentrations indicated in the figure legends. The volume injected was estimated to be 1-2x10^-14 liters (Graessmann and Garessmann, 1983). Microinjection needles were pulled from glass capillaries (Clark Electromedical Instruments, Reading, UK) on an automatic pipette puller (David Kopf Instruments, Tujunga, CA, USA, or Zeitz Instruments, Augsburg, Germany).

Immunofluorescent staining

Microinjected cells were washed three times with PBS, fixed in 4% ice-cold paraformaldehyde in PBS for 15 minutes, permeabilized for 20 minutes in 0.2% Triton X-100 in PBS, and blocked for at least 1 hour in 10% FCS in PBS to reduce nonspecific staining. T antigen was visualized by staining for 1 hour with an antibody mix containing mouse monoclonal antibodies Pab 101 (Gurney et al., 1980), Pab 221 (Mole et al., 1987), Pab 416 and 419 (Harlow et al., 1981) (10 µg/ml each) followed by FITC-conjugated second antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:50) for 1 hour. Antibodies were diluted in 10% FCS in PBS; the incubations were carried out at room temperature. After each antibody incubation, cells were washed three times quickly and then another three times for 10 minutes each. After the last wash step, the coverslips were air-dried, mounted in 90% glycerol containing 0.1 mg/ml paraphenylenediamine in PBS (Johnson and Nogueria Araujo, 1981), and viewed on an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) using a x63 objective.

RESULTS

Nuclear protein import is temperature-sensitive in tsBN2 cells at the restrictive temperature

The hamster cell line tsBN2 expresses a temperature-sensitive RCC1, resulting in thermosensitive export of mRNA from the nucleus into the cytoplasm (Amberg et al., 1993; Kadowaki et al., 1993; data not shown). To test if this mutation also influences nuclear protein import, tsBN2 cells and the parental baby hamster kidney cells, BHK21, were preincubated for 6 hours at the permissive (33.5°C) or nonpermissive (39.5°C) temperature. Purified SV40 large T antigen was microinjected into the cytoplasm and incubation was continued for another 5 to 120 minutes. T antigen import into the nucleus was detected by immunofluorescent staining of the fixed, permeabilized cells by using a monoclonal antibody mix, followed by a fluorophore-coupled second antibody. Fig. 1 shows typical staining patterns for each cell line at 60 minutes after microinjection. In tsBN2 cells at the permissive temperature, T antigen was efficiently imported into the nucleus, but at the nonpermissive temperature nuclear import was markedly impaired (Fig. 1A,B). At the permissive temperature, all of the cells showed exclusively nuclear staining (cells indicated by a small arrow in Fig. 1B). At the nonpermissive temperature, in contrast, many cells showed staining in both the nucleus and cytoplasm (indicated by a large arrow). In the parental control cell line BHK21, T antigen import was equally efficient at both temperatures (Fig. 1C,D).

The defect in nuclear protein import became evident in tsBN2 cells about 2 hours after the shift to the nonpermissive temperature and was maximal after about 3 hours at the restric-
tive temperature (data not shown). When the cells were shifted back to the permissive temperature, nuclear protein import was increased within one hour and restored to normal rates by 3 hours after shift-down (data not shown).

To quantitatively evaluate the ability of tsBN2 cells to import T antigen, the cells containing only nuclear (N) and only cytoplasmic (C) T antigen were counted, expressed as a fraction of the total number of cells containing T antigen, and plotted as a function of the time after microinjection (Fig. 2A,B). Nuclear import of T antigen was complete within 30 minutes at the permissive temperature, but at the nonpermissive temperature, import activity was strongly reduced. Even at 120 minutes after injection, only 10% of the cells showed exclusively nuclear staining. In the rest of the cells, T antigen was cytoplasmic or distributed between the nucleus and cytoplasm. Import of a tagged retinoblastoma tumor suppressor protein (Rb), which carries a bipartite NLS in the C-terminal region and an additional sequence encoded in exon 22 that enhances its rate of import (Zacksenhaus et al., 1993), was complete within 15 minutes after injection into the cytoplasm at the permissive temperature, but progressed much more slowly at the nonpermissive temperature (data not shown). Import of human serum albumin covalently coupled to multiple T antigen NLS peptides and microinjected into the cytoplasm of tsBN2 cells was also thermosensitive (data not shown). In contrast, nuclear import of T antigen in BHK21 cells was complete within 30 minutes at both temperatures (Fig. 2C,D). These results demonstrate that RCC1 is required for efficient nuclear protein import in vivo, confirming and extending a recent report (Tachibana et al., 1994).

Nuclear import of U1snRNPs is not dependent on RCC1

The thermosensitive nuclear protein import in tsBN2 cells could arise through an indirect mechanism in which the RNA that is blocked in export at the nonpermissive temperature accumulates at the nucleoplasmic side of the NPC and clogs the pores. The findings that nuclear import of proteins and snRNPs utilized different receptors and that a block in nuclear protein import failed to block snRNP import (Michaud and Goldfarb, 1992; van Zee et al., 1993) provide an opportunity to distinguish between these possibilities. To test whether the NPCs are blocked at the nonpermissive temperature, we compared nuclear import of fluorescently labelled U1snRNPs microinjected into tsBN2 cells at both temperatures. After incubation for various times, the cells were fixed, mounted and evaluated. As shown in Fig. 3A,B, U1snRNP import was equally efficient at the permissive and nonpermissive temperatures. To confirm this observation, quantitative evaluation of U1snRNP import kinetics was performed (Fig. 3C,D). At each time point, the injected cells were classified according to the nucleo-cytoplasmic distribution of U1snRNP, as described in the figure legend. The import rate was the same at both temperatures, arguing that the NPC in tsBN2 cells are competent for import at both temperatures. These results are consistent with a recent report that tRNA export is also not thermosensitive in tsBN2 cells (Cheng et al., 1995).

Cytoplasmic injection of Ran-GTP or Ran-GDP alleviates the temperature-sensitive defect in nuclear protein import

The function of RCC1 as a guanine nucleotide exchange factor for Ran in vitro (Bischoff and Ponstingl, 1991a,b) and the essential role of Ran and GTP in nuclear protein import in vitro (Moore and Blobel, 1993; Melchior et al., 1993) suggested that the thermosensitivity of nuclear protein import in tsBN2 cells could arise through a shortage of Ran-GTP in the cytoplasm. To test this idea, we attempted to rescue nuclear protein import in tsBN2 cells at the nonpermissive temperature by microinjecting additional Ran-GTP into the cytoplasm.

Recombinant Ran was purified from extracts as described in Materials and Methods and charged with nucleotide by incubating it with either GTP or GDP. Its purity was verified by denaturing gel electrophoresis and Coomassie staining (data not shown). Over 95% of the nucleotide bound to Ran-GTP was GTP; the rest being GDP (data not shown), consistent with

Figure 1. Nuclear import of T antigen is temperature-sensitive in tsBN2 cells. TsBN2 cells (A,B) and BHK21 cells (C,D) were seeded on coverslips and incubated for 2 days prior to injection. Six hours before microinjection, the cells were shifted to the permissive temperature (33.5°C, A,C) or to the nonpermissive temperature (39.5°C, B,D). The cells were microinjected with purified SV40 large T antigen (1 mg/ml) and fixed 1 hour later. T antigen was visualized by immunofluorescence staining and the coverslips were mounted and photographed. Examples of cells containing exclusively nuclear T antigen (small arrow), nuclear and cytoplasmic T antigen (filled arrowheads), and exclusively cytoplasmic T antigen (large arrow) are indicated. Bar, 50 μm.
the slow intrinsic rates of GTP hydrolysis and guanine nucleotide exchange previously reported for Ran (Bischoff and Ponstingl, 1991b). GDP was the only nucleotide bound to Ran-GDP (not shown).

The effect of Ran-GTP on nuclear protein import in tsBN2 cells was then measured at the permissive and nonpermissive temperatures. SV40 large T antigen mixed with Ran-GTP, or with GTP alone in Ran elution buffer as a control, was injected into the cytoplasm and incubation was continued for another 60 minutes at either temperature. The cells were then fixed, permeabilized, blocked, and stained by immunofluorescence (Fig. 4). At the permissive temperature, T antigen import was equally efficient in cells injected with Ran-GTP and Ran buffer (Fig. 4A,B). T antigen import in the presence of Ran buffer was not detectably different at either temperature from that in Ran buffer with GTP (data not shown). However, in the cells incubated at the restrictive temperature, nuclear import was clearly stimulated by Ran-GTP (Fig. 4F) compared to the control performed with Ran buffer (Fig. 4E). Unexpectedly, when Ran-GDP was mixed with T antigen, it also stimulated nuclear protein import at the nonpermissive temperature (Fig. 4G).

To confirm these results, nuclear import of T antigen in the

Fig. 2. Comparison of nuclear import kinetics of T antigen in a mutant and a normal hamster cell line. T antigen import was evaluated quantitatively as a function of the time after microinjection into tsBN2 (A,B) and BHK21 (C,D) cells at the permissive (A,C) and nonpermissive (B,D) temperatures as in Fig. 1. At each time point, the number of cells stained only in the nucleus (N) or cytoplasm (C) was counted and divided by the total number of cells stained. Cells with both nuclear and cytoplasmic staining were included in the total. The total number of cells counted per time point was 386-455 in 4 independent experiments for tsBN2 and 95-293 in 2 independent experiments for BHK21.

Fig. 3. Nuclear import of U1snRNPs is not dependent on RCC1 in tsBN2 cells. (A,B) TsBN2 cells were seeded on coverslips and incubated for 2 days prior to injection. Six hours before microinjection, the cells were shifted to the permissive temperature (33.5°C, A) or to the nonpermissive temperature (39.5°C, B). The cells were microinjected with fluorescently labelled U1snRNPs (1 mg/ml) and fixed 1 hour later. The coverslips were mounted and photographed. Examples of cells containing predominantly nuclear U1snRNPs (arrow), and predominantly cytoplasmic U1snRNPs (arrowhead) are indicated. (C,D) Nuclear import kinetics of U1snRNP were evaluated quantitatively as a function of time after cytoplasmic microinjection into tsBN2 cells at the permissive and nonpermissive temperatures as in A and B. At each time point, the number of cells stained predominantly in the nucleus (N>>C) or predominantly in the cytoplasm (N<<C) was counted and divided by the total number of cells stained. The total number of cells counted per time point was 51-166 in 3 independent experiments.
presence of Ran-GTP and Ran-GDP was quantitatively evaluated as a function of time after microinjection into the cytoplasm. As a control, nuclear import of T antigen mixed with Ran elution buffer was analyzed in parallel (Fig. 4D,H). At the permissive temperature, the import kinetics of T antigen in the presence and absence of either Ran-GTP or Ran-GDP were similar to those in the control cells (Fig. 4D). In contrast, both Ran-GTP and Ran-GDP accelerated the import of T antigen into the nucleus at the nonpermissive temperature. The fraction of cells containing only nuclear T antigen (Fig. 4H) increased more rapidly in cells injected with Ran than in the control. The kinetics of stimulation of nuclear import by equal concentrations of Ran-GTP and Ran-GDP were not significantly different (Fig. 4H). These results confirm that cytoplasmic injection of Ran in either the GTP- or GDP-bound form suppressed the thermosensitivity of nuclear protein import in tsBN2 cells with equal efficiency.

**Fig. 4.** Effects of Ran-GTP and Ran-GDP on nuclear protein import in vivo. TsBN2 cells were incubated at the permissive (A,B,C) and nonpermissive temperature (E,F,G) as described in Fig. 1 and microinjected in the cytoplasm with T antigen mixed with (A,E) Ran elution buffer containing GTP, (B,F) Ran-GTP, or (C,G) Ran-GDP. The final concentrations injected were 0.5 mg/ml T antigen, and 4 mg/ml Ran-GTP or Ran-GDP. The cells shown here were fixed 60 minutes after microinjection, quantitatively evaluated as in Fig. 2 and the percentage of cells with exclusively nuclear staining (N) was plotted (D,H). Similar results were obtained in 3-6 experiments with 59-261 cells per time point in each experiment. Bar, 50 μm.

**Nuclear protein import is severely inhibited by the GTPase-deficient mutant Ran Q69L at both the permissive and nonpermissive temperatures**

The ability of Ran-GDP to stimulate nuclear protein import in the absence of RCC1 was unexpected and raised the question of whether both Ran-GDP and Ran-GTP might be active in import. To test this idea, we reasoned that if only Ran-GTP were active in nuclear protein import, then a mutant Ran defective in import should block import only in the GTP-bound form. In the absence of RCC1, i.e. at the nonpermissive temperature for tsBN2 cells, the GDP-bound form should not be efficiently converted to the GTP-bound form and should not block import. In *Saccharomyces cerevisiae*, a dominant GTPase-defective mutant Ran (Gsp1-G21V) (Belhumeur et al., 1993), assumed to be trapped in the GTP-bound form, was shown to block nuclear protein import when overexpressed in vivo (Schlenstedt et al., 1995). To further investigate the role of Ran-GDP in nuclear protein import, we have used another GTPase-deficient mutant RanQ69L (Ren et al., 1993; Bischoff et al., 1994; Klebe et al., 1995). Both nucleotides have been shown to bind efficiently to RanQ69L and both forms of RanQ69L can be used as substrates by RCC1 in vitro (Klebe et al., 1995).

T antigen was mixed with Ran elution buffer as a control, or with purified RanQ69L-GTP or -GDP, and microinjected into the cytoplasm of tsBN2 cells at the permissive or restrictive temperature. After incubation for another 60 minutes, the cells were fixed and stained by immunofluorescence (Fig. 5). Nuclear import of T antigen was blocked by RanQ69L-GTP at both temperatures (Fig. 5B,F) and kinetic analysis revealed that
T antigen import remained blocked for at least 2 hours after microinjection (Fig. 5D,H). The results are consistent with the requirement for Ran GTPase in nuclear protein import (Schlenstedt et al., 1995; Melchior et al., 1995; Corbett et al., 1995) and indicate that RanQ69L-GTP interfered with the import activity of the endogenous Ran at the permissive temperature. However, injection of RanQ69L-GDP also blocked T antigen import at both temperatures (Fig. 5C,G) and inhibition was quantitatively very similar to that observed with RanQ69L-GTP (Fig. 5D,H). Since RCC1 activity could convert RanQ69L-GDP to the GTP-bound form at the permissive temperature, it is perhaps not surprising that the GDP-bound form was just as inhibitory as the GTP-bound form. At the nonpermissive temperature, on the other hand, either RanQ69L-GDP was converted to the GTP-bound form in the apparent absence of RCC1 or it inhibited import directly.

Nuclear protein import is inhibited by mutant RanT24N-GDP at the permissive temperature

To pursue the possible role of Ran-GDP and RCC1 in nuclear protein import, we tested the mutant RanT24N. This mutant binds guanine nucleotides with reduced affinity, but binds to GDP several orders of magnitude better than to GTP, and also binds tightly to RCC1, thereby inactivating it (Dasso et al., 1994; Klebe et al., 1995). RanT24N reduced nuclear protein import into Xenopus nuclei assembled in egg extracts by less than 50%, whereas depletion of RCC1 in the extracts essentially abolished nuclear protein import (Kornbluth et al., 1994; Dasso et al., 1994). Based on our findings and those of Tachibana et al. (1994) that RCC1 is important for nuclear protein import in tissue culture cells in vivo, one might expect RanT24N to inhibit import in tsBN2 cells at the permissive temperature by binding to RCC1 and preventing GTP-GDP exchange on endogenous Ran. However, based on the results with Xenopus nuclear import in egg extracts, one might expect RanT24N to have little or no effect on import.

To distinguish between these two possibilities, T antigen, mixed either with the Ran mutant RanT24N-GDP or with buffer as a control, was microinjected into the cytoplasm of tsBN2 cells at the permissive and nonpermissive temperatures. After 60 minutes of further incubation, the cells were fixed and stained by immunofluorescence. Fig. 6A,B shows that nuclear protein import was inhibited by RanT24N at the permissive temperature. The results at the nonpermissive temperature showed no detectable increase in T antigen import in the presence of RanT24N-GDP compared with the control (compare Fig. 6D,E), implying that RanT24N-GDP is not

Fig. 5. Inhibition of nuclear protein import by mutant Q69LRan in the GTP- and GDP-bound forms. TsBN2 cells incubated at the permissive (A,B,C) or nonpermissive temperature (E,F,G) were microinjected in the cytoplasm with T antigen (0.5 mg/ml final concentration) mixed with (A,E) Ran buffer, (B,F) Q69LRan-GTP (2 mg/ml final concentration), or (C,G) Q69LRan-GDP (2 mg/ml final concentration). The cells were fixed at various times after injection, and T antigen was visualized by immunofluorescence. The cells shown here were fixed 60 minutes after microinjection. Import was evaluated as a function of time after injection as described in Fig. 2 and the fraction of cells showing only nuclear staining was plotted against time (D,H). The experiments were performed 2-5 times with 51-177 cells counted per time point in each experiment. Bar, 50 μm.
active in nuclear protein import. Quantitative evaluation of kinetic experiments confirmed this conclusion and suggested that it reduced import even further (Fig. 6C,F).

**DISCUSSION**

**RCC1 is required for efficient nuclear protein import in vivo**

Using the mutant cell line tsBN2, we have demonstrated that import of SV40 T antigen, the tumor suppressor protein Rb, and a serum albumin-NLS peptide conjugate is thermosensitive. In the parental cell line BHK21, efficient nuclear import of all three substrates was maintained at the nonpermissive temperature, confirming that RCC1 function plays an important role in nuclear protein import in intact cells (Figs 1, 2; Tachibana et al., 1994). The NPC retain their capacity for nucleo-cytoplasmic exchange of macromolecules in the absence of RCC1, since nuclear import of U1snRNP (Fig. 3) and export of tRNA (Cheng et al., 1995) were not impaired at the nonpermissive temperature. These results argue that occlusion of the NPC by unexported RNA that accumulates in the absence of RCC1 is not responsible for inhibition of nuclear protein import and that the mechanism of inhibition is probably more direct.

**The role of RCC1 and Ran in nuclear protein import**

Since the only biochemical activity of RCC1 known so far is its Ran-specific guanine nucleotide exchange activity (Bischoff and Ponstingl, 1991a,b), and since Ran and GTP are required for nuclear protein import in digitonin-permeabilized cells (Moore and Blobel, 1993; Melchior et al., 1993), we reasoned that RCC1 might be needed to provide Ran-GTP in the cytoplasm for protein import. Consistent with this notion, microinjection of Ran-GTP, but not GTP alone, into the cytoplasm of tsBN2 cells at the nonpermissive temperature restored efficient nuclear protein import. We estimate that about 10-20·10^5 Ran molecules were injected into each cell in the experiments shown here. When Ran was purified by a more rapid procedure, similar stimulation of protein import was attained with half as much Ran-GTP (I. Boche, A. Dickmanns, and E. Fanning, unpublished data). The amount of Ran injected into each tsBN2 cell is a small fraction of the endogenous Ran (10^7 molecules per HeLa cell; Bischoff and Ponstingl, 1991a). Based on this estimate, the observed stimulation of import was probably not due to a significantly increased total concentration of Ran in the cells, which is thought to account for the multicopy Ran-mediated suppression of certain conditional RCC1 mutations in yeast (Matsumoto and Beach, 1991; Belhumeur et al., 1993; Kadokawa et al., 1993; Sazer and Nurse, 1994). Thus one possible interpretation of our data is

![Image](image_url)
that the increased concentration of cytoplasmic Ran-GTP after microinjection was directly responsible for stimulation of nuclear protein uptake.

Several observations are not accounted for in this simple interpretation. The first is that Ran-GDP stimulated nuclear protein import with an activity equal to that of Ran-GTP in the absence of RCC1, although a GTPase-defective mutant Ran in the GDP-bound form did not. On the contrary, RanQ69L-GDP inhibited import at both temperatures as strongly as did RanQ69L-GTP. Secondly, although the endogenous Ran in intact tsBN2 cells is localized primarily in the nucleus at the permissive temperature, increased amounts were found in the cytoplasm at the nonpermissive temperature, presumably in the GDP-bound form (Ren et al., 1993; Tachibana et al., 1994). Indeed, it has been speculated that cytoplasmic Ran-GDP acts as an inhibitor of nuclear protein import in tsBN2 cells (Tachibana et al., 1994). While our results demonstrate that Ran-GDP is not an inhibitor of import, the mechanism by which it stimulates import in tsBN2 cells in the absence of RCC1 is not clear.

A second possible interpretation of our results that could also account for these observations is that intact tsBN2 cells retain enough RCC1 activity at the nonpermissive temperature to generate Ran-GTP when supplied with even a slightly elevated concentration of wild-type Ran by microinjection, i.e. that the mutation is leaky. In this case, the Ran-GDP would be converted to Ran-GTP by trace amounts of RCC1 and could stimulate import. Similarly, RanQ69L-GDP would undergo RCC1-catalyzed nucleotide exchange and inhibit import. This possibility could explain the incomplete shutdown of nuclear protein import at the nonpermissive temperature (Tachibana et al., 1994; this report). It also predicts that it should be relatively unimportant for suppression of the thermosensitive import defect whether the additional Ran is supplied in the GTP-, GDP-, or unbound form. In keeping with this prediction, preliminary results show that microinjection of Ran not loaded with nucleotide also suppressed the thermosensitivity of nuclear protein uptake.

A third possibility consistent with our results would be that Ran-GDP is efficiently converted to Ran-GTP in tsBN2 cells through a mechanism independent of RCC1 and can then directly stimulate nuclear protein import. In this case, the small increase in concentration after microinjection of Ran-GDP would be required to accelerate this conversion and compensate for the loss of RCC1. RanQ69L-GDP could be converted to the GTP-bound form by the same mechanism, thereby blocking protein import. The weak guanine nucleotide binding affinity of RanT24N might be expected to inhibit such a novel exchange activity in the same way that it inhibits RCC1. Although a search for a novel Ran-nucleotide exchange activity in soluble cytoplasmic extracts revealed only traces of RCC1 thought to have leaked from the nucleus (Bischoff et al., 1995a), this does not rule out the possible existence of a novel conversion mechanism. For example, cytoplasmic Ran-GDP could interact with the karyophilic protein-NLS receptor complex after it has docked at the pore, thereby triggering GDP-GTP exchange in situ by the docked complex, other soluble factors, the pore itself, or some combination of these.

In situ conversion of Ran-GDP to Ran-GTP at the NPC might be difficult to detect in soluble extracts. Clearly, further work will be required to understand the mechanism by which Ran-GDP suppresses the thermosensitivity of nuclear protein import in tsBN2 cells and the role of RCC1 in nucleo-cytoplasmic trafficking.

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