sufficiently long for the regeneration of an atmospheric haze cold enough to condense O_2 after an eclipse. The present laboratory results provide a rationale for assessing the possibility of a variable sublimation-driven atmosphere and haze that are cold enough to condense O_2.

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Requirement of Guanosine Triphosphate–Bound Ran for Signal-Mediated Nuclear Protein Export

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A leucine-rich nuclear export signal (NES) allows rapid export of proteins from cell nuclei. Microinjection studies revealed a role for the guanosine triphosphatase (GTPase) Ran in NES-mediated export. Nuclear injection of a Ran mutant ($Thr^{24} \rightarrow Asn$) blocked protein export but not import, whereas depletion of the Ran nucleotide exchange factor RCC1 blocked protein import but not export. However, injection of Ran GTPase-activating protein (RanGAP) into RCC1-depleted cell nuclei inhibited export. Coinjection with Ran mutants insensitive to RanGAP prevented this inhibition. Therefore, NES-mediated protein export appears to require a Ran-GTP complex but does not require Ran-dependent GTP hydrolysis.

Bidirectional movement of proteins across the nuclear membrane occurs through the nuclear pore complex. Import of nuclear proteins containing a nuclear localization signal (NLS) requires the small GTPase Ran and associated proteins (*1*). The process of nuclear protein export is less well understood, but the recent identification of NESs suggests that NES-specific receptors are likely involved. Related NESs have been identified in Ran binding protein 1 (RanBP1) (*2*), protein kinase A inhibitor, mitogen-activated protein kinase kinase, the yeast protein Gle1p, and the retroviral proteins Rev and Rex (*3*).

To examine the role of Ran in nuclear protein export, we constructed an export substrate, GGNES, that contained glutathione-S-transferase (GST) attached to green fluorescent protein (GFP) and the RanBP1 NES (Lys-Val-Ala-Glu-Lys-Leu-Glu-Ala-Leu-Ser-Val-Arg, residues 178 to 189) (*4*). At 55 kD, this substrate is too large to diffuse passively through the nuclear pores within the experimental time frame. GGNES and GG (a GST-GFP construct lacking the NES) were each expressed in *Escherichia coli*, purified, and injected into nuclei of baby hamster kidney cells (BHK21); fluorescent dextran marked the injection site (*5*). After a 60-min incubation, the GG and GGNES proteins were visualized by epifluorescence microscopy. The GG control remained nuclear after injection, but the GGNES substrate was exported to the cytoplasm (Fig. 1A). To determine the rate of GGNES export, we incubated injected cells for various intervals before fixation and analysis (*6*). GGNES export was essentially complete by 15 min (Fig. 1B).

To further examine the role of Ran in NES-dependent nuclear protein export, we coinjected selected Ran mutants with GGNES. Mutants were chosen that were expected to display dominant phenotypes. A mutant in which Gly^{19} was replaced by Val (Ran G19V) is insensitive to the Ran nucleotide exchange factor, RCC1, and to the Ran GTPase-activating protein, RanGAP; it is predominantly in an active, GTP-bound form in BHK21 cells (*7*). Ran T24N ($Thr^{24} \rightarrow Asn$) does not bind nucleotide stably and exhibits increased affinity for RCC1 (*7, 8*). Ran E46G ($Glu^{46} \rightarrow Gly$) has a mutation in the effector domain, which is thought to mediate interactions with downstream target proteins. The E46G mutant is insensitive to RanGAP but undergoes RCC1-mediated nucleotide exchange, which suggests that it is GTP-bound in the cell (*7*).

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Neither Ran G19V nor Ran E46G blocked nuclear export when coinjected into nuclei with GGNES (Fig. 2A and Table 1). Ran T24N, however, blocked the export of GGNES. The onset of inhibition was rapid, because the half-time for GGNES export is <15 min; this inhibition appeared to be persistent, because no export was detected after incubation of the cells for 60 min. The effect was specific for this Ran mutant; an analogous mutant of a different GTPase, Rab3A T36N, had no effect.

The inhibitory effect of Ran T24N on GGNES nuclear export may be attributable to sequestration and inhibition of the RCC1 exchange factor (*7*, *8*), which depletes the nucleus of the Ran-GTP complex. Ran-GTP is required for release of import complexes after translocation through nuclear pores (*9*), and Ran-GTP depletion might trap import complexes in the pores and inhibit export by occlusion. Ran T24N inhibits NLS-mediated nuclear protein import when injected into the cytoplasm (*10*).

Possible effects on nuclear import were examined in two ways. First, we transfected cells with a glucocorticoid receptor–GFP construct (GR-GFP). This fusion protein contains an NLS but remains cytoplasmic until exposed to the agonist dexamethasone (*10*). Injection of Ran T24N into the nucleus did not inhibit the dexamethasone-induced nuclear translocation of GR-GFP. Import was observed after 15 to 30 min (Fig. 2B), a period within which GGNES export is inhibited (Fig. 1).

Second, we investigated whether the inhibition of nuclear export by Ran T24N could be reversed by a dominant activated mutant of Ran, which might release trapped import complexes. We injected GGNES into nuclei together with an equimolar mixture of Ran T24N and Ran G19V. GGNES was not exported (*11*); therefore, export does not appear to be inhibited by the arrest of transport complexes involved in the NLS-mediated import of the glucocorticoid receptor. We have not excluded the possibility that other import complexes are trapped at the nuclear pore by Ran T24N. However, other Ran-dependent import pathways have not yet been described.

RCC1 may play a direct role in nuclear protein export, and this role may be inhibited by the formation of a Ran T24N–RCC1 complex. We therefore studied tsBN2 cells, which are derived from BHK21 cells and have a temperature-sensitive RCC1 allele (*12*). When tsBN2 cells were held at the restrictive temperature for 3 or 6 hours, the amount of cellular RCC1 was substantially reduced (Fig. 3A). Concomitant addition of cycloheximide to prevent resynthesis did not further reduce RCC1 concentrations but prevented reexpression during subsequent manipulations (*13*).

To test nuclear export capacity, we treated tsBN2 cells with cycloheximide at either the permissive or restrictive temperature for 3 hours and then injected their nuclei with GGNES. GGNES was exported into the cytoplasm regardless of temperature (Fig. 3B). However, at 39.5°C, NLS-dependent nuclear import was arrested (Fig. 3C). Cycloheximide treatment had no effect on GR-GFP import (*11*). Because NES-

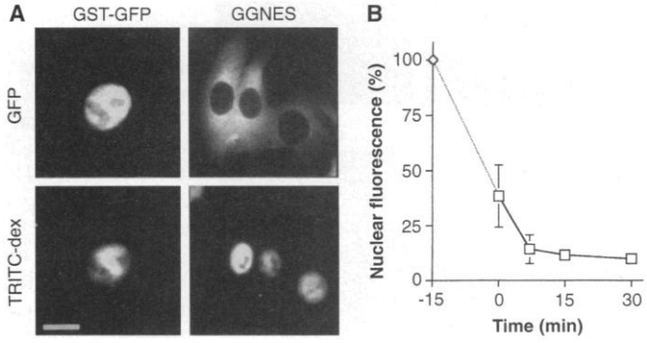
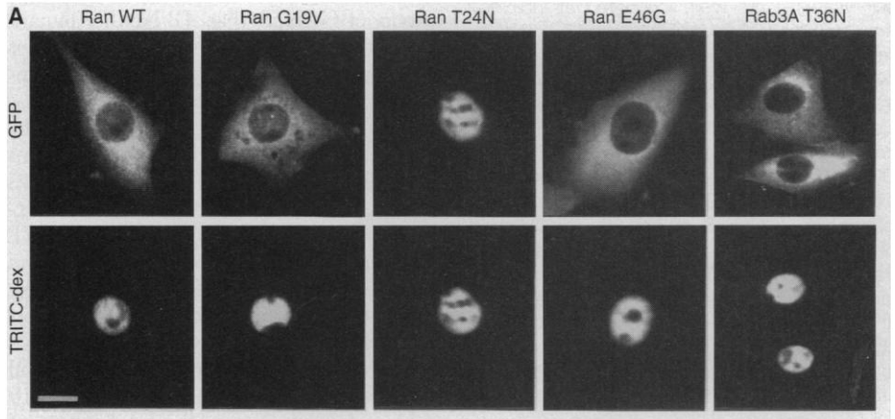


Fig. 1. NES-mediated nuclear protein export. (**A**) Microinjection of BHK21 cells with export substrates. Cells were injected with TRITC-dextran (TRITC-dex) plus GG (GST-GFP) or GGNES and incubated at 37°C for 60 min before fixation (*10*). In this and other figures, the TRITC-dex panels show the injection sites. Scale bar, 25 μm. (**B**) Time course of GGNES export. BHK21 nuclei were injected with TRITC-dextran plus GGNES as in (A) and were incubated at 37°C for various periods before fixation. Analysis was done as described (*6*); n = 10 to 13.



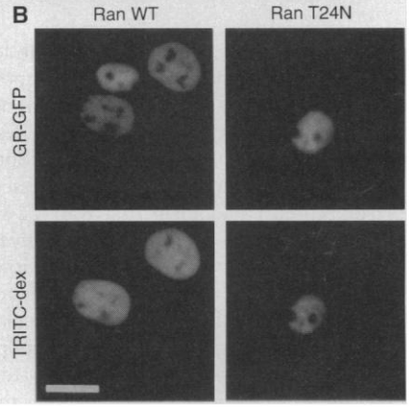


Fig. 2. Inhibition of nuclear protein export, but not import, by nuclear Ran T24N. (**A**) Export of GGNES from BHK21 cell nuclei that were also injected with Ran mutant proteins. Nuclei were injected with TRITC-dextran, GGNES, and the indicated protein. Cells were treated as in Fig. 1A. Scale bar, 25 μm. (**B**) Nuclear import of GR-GFP. Cells were transfected with 2 μg of pK7-GR-GFP as described (*10*). Two days after transfection, nuclei of transfected cells were injected with TRITC-dextran plus Ran mutant. Dexamethasone (1 μM) was added after 15 min, and cells were returned to 37°C for a further 15 min. GR-GFP protein was visualized by confocal microscopy. Scale bar, 25 μm.

Table 1. Effect of Ran mutant proteins on GGNES export (n = 50 for each mutant). Cell nuclei were injected and processed as in Fig. 2A. Injected cells were scored by eye, by two observers, for distribution of GGNES. Only cells that were injected in the nucleus, as determined by TRITC-dextran localization, were scored. "Mixed" indicates that the GGNES was equally distributed between nucleus and cytoplasm.

Injection	Nuclear (%)	Mixed (%)	Cytoplasmic (%)
GGNES	0	0.4	99.6
Ran WT	0	1.8	98.2
Ran G19V	0.8	5.4	93.8
Ran T24N	89.4	6.7	3.9
Ran E46G	0	0.9	99.1
Rab3A T36N	0	3.7	96.3

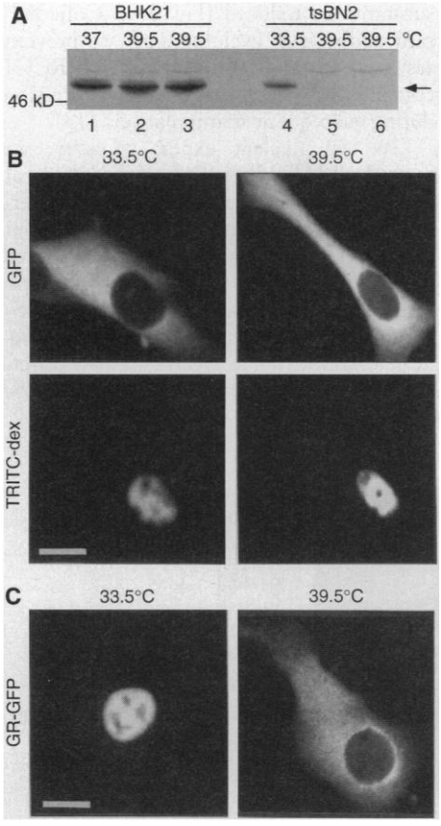


Fig. 3. Export of GGNES occurs in tsBN2 cells in which nuclear import has been blocked. (**A**) Protein immunoblot for RCC1 in BHK21 and tsBN2 cell lysates. Cells were incubated at indicated temperatures for 3 hours (lanes 2 and 5) or 6 hours (lanes 1, 3, 4, and 6). Cell lysates were separated by polyacrylamide gel electrophoresis (10% gel), transferred to nitrocellulose, and blotted with polyclonal antibody to RCC1 at 1:2000 dilution as described (*2*). Arrow indicates RCC1. (**B**) Export of GGNES in tsBN2 cells. The tsBN2 cells were placed at 33.5° or 39.5°C for 3 hours before injection with GGNES. Scale bar, 25 μm. (**C**) Import of GR-GFP in tsBN2 cells. The tsBN2 cells were transfected with pK7-GR-GFP (*10*) and maintained at 33.5°C. After 2 days, cells were treated at 33.5° or 39.5°C for 3 hours with 35.5 μM cycloheximide. Dexamethasone (1 μM) was added for 15 min. Cells were analyzed by confocal microscopy. Scale bar, 25 μm.

mediated nuclear export can occur under conditions in which nuclear protein import is blocked, whereas NLS-mediated import can occur when export is inhibited, these processes appear not to be coupled. Nonetheless, Ran participates in both processes.

It appeared paradoxical that Ran T24N could arrest nuclear protein export even though depletion of RCC1 could not. However, RCC1 is an efficient exchange factor (*14*), so tsBN2 cells may retain sufficient nuclear Ran-GTP to support nuclear export, even under restrictive conditions. To test this possibility, we injected nuclei with the murine RanGAP, Fug1, which would deplete nuclei of residual GTP-bound Ran. Nuclear injection of GST-Fug1 into tsBN2 cells at 39.5°C efficiently blocked export of GGNES (Fig. 4). Under these conditions, the diffusive export of a small (10 kD) fluorescent dextran was unimpeded, which suggested that the nuclear pores had not been occluded (*11*). Injection of Ran T24N into the RCC1-depleted tsBN2 cell nuclei also blocked GGNES export, which was not rescued by addition of the activated mutant, Ran G19V (*11*).

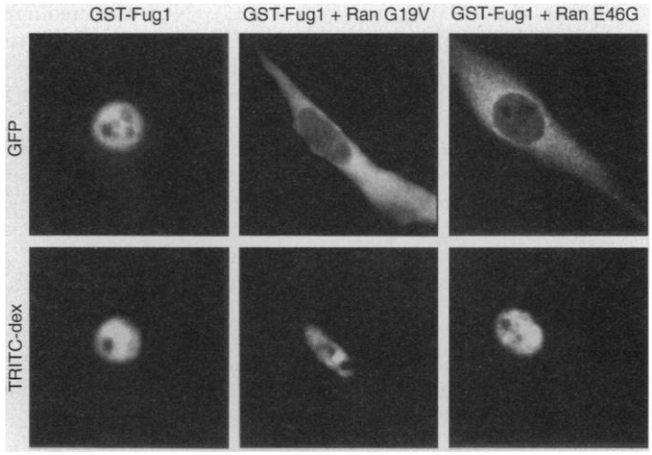


Fig. 4. Requirement of NES export for Ran-GTP but not Ran-dependent GTP hydrolysis. The tsBN2 cells at 39.5°C were treated with cycloheximide for 3 hours, then injected with GGNES and TRITC-dextran. Where indicated, GST-Fug1 (RanGAP) plus Ran G19V or Ran E46G were also injected. Cells were returned to 39.5°C for 1 hour before analysis (*5*).

We also coinjected GST-Fug1 with Ran G19V or Ran E46G. Both mutants restored export of GGNES (Fig. 4). Neither mutant has a high affinity for Fug1 (*11*), and it is unlikely that they act by binding to and inhibiting the GST-Fug1. Therefore, nuclear Ran-GTP appears to be necessary for NES-mediated protein export, but Ran- dependent GTP hydrolysis may not be required for this process. Additionally, RCC1-catalyzed exchange between Ran-GDP and Ran-GTP may trigger release of some other factor, and the interaction of Ran-GTP with this factor would then permit operation of the export machinery. Ran T24N, which binds RCC1 with high affinity (*7*, *8*), may also bind to and sequester the export factor, inhibiting both nucleotide exchange on Ran and release of this additional export factor. Coinjection of an activated Ran mutant would not rescue the Ran T24N–induced export defect because this second factor would not be available. Dis3, which interacts with both RCC1 and Ran in the nucleotide-free state (*15*), is a candidate for this export factor.

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4. Export substrates GST-GFP (GG) and GST-GFP-NES (GGNES) were made as described (*2*). Ran proteins were generated as GST fusions and cleaved with thrombin as described (*16*). Proteins were dialyzed into injection buffer [10 mM $NaPO_4$ (pH 7.2), 70 mM KCl, and 1 mM $MgCl_2$], divided into aliquots, and stored at −80°C.
5. BHK21 cells were maintained in Dulbecco's minimal essential medium with calf serum (10% v/v) at 37°C, 5% CO_2. The tsBN2 cells (*12*) were maintained at their permissive temperature, 33.5°C. Injection mixes included tetramethyl rhodamine isothiocyanate–coupled (TRITC) dextran (1.6 mg/ml, Sigma), GG or GGNES (1 mg/ml), and other protein (1 mg/ml) as indicated, in injection buffer. Injections were done over 15 min. Cells were returned to 33.5°, 37°, or 39.5°C as indicated for 60 min before fixation (*10*). Cells were mounted and analyzed on a Bio-Rad MC1000 confocal system attached to an Olympus BX50 microscope with a 40× objective lens.
6. Fluorescence distribution within cells was quantitated with Comos software on the Bio-Rad MC1000 confocal system. Pixel values were gathered by accumulation to peak intensity. Percentage of nuclear fluorescence was calculated as [Σnuclear pixels/ Σ(nuclear + cytoplasmic pixels)] × 100.
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