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Gabriel Schlenstedt
Harvard University

Claudio Saavedra
Dartmouth College

Jonathan D. Loeb
Massachusetts Institute of Technology

Charles N. Cole
Dartmouth College

Pamela A. Silver
Harvard University

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The GTP-bound form of the yeast Ran/TC4 homologue blocks nuclear protein import and appearance of poly(A)$^+$ RNA in the cytoplasm

GABRIEL SCHLENSTEDT*, CLAUDIO SAAVEDRA†, JONATHAN D. J. LOEB‡, CHARLES N. COLE†, AND PAMELA A. SILVER*§

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School and Dana–Farber Cancer Institute, 44 Binney Street, Boston, MA 02115; †Department of Biochemistry, Dartmouth University Medical School, Hanover, NH 03755; and ‡Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142

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ABSTRACT **Ran/TC4, a Ras-like GTP-binding protein, and its nucleotide exchanger, RCC1, have been implicated in control of protein movement into the nucleus and cytoplasmic accumulation of mRNA. *Saccharomyces cerevisiae* contains two homologues of the mammalian Ran/TC4, encoded by the *GSP1* and *GSP2* genes. We have constructed yeast strains that overproduce either wild-type Gsp1 or a form of Gsp1 with glycine-21 converted to valine (Gsp1-G21V), which we show stabilizes the GTP-bound form. Cells producing Gsp1-G21V have defects in localization of nuclear proteins; nuclear proteins accumulate in the cytoplasm following galactose induction of Gsp1-G21V. Similarly, cells producing Gsp1-G21V retain poly(A)$^+$ RNA in their nuclei. These findings suggest that hydrolysis of GTP by Ran/TC4 is necessary for proper import of proteins into the nucleus and appearance of poly(A)$^+$ RNA in the cytoplasm.**

Movement of proteins and RNAs across the nuclear envelope via the nuclear pores is rapid, specific, and highly regulated. Proteins destined for the nucleoplasm often contain short stretches of amino acids, termed nuclear localization sequences (NLSs), that are sufficient to direct them to the nucleus (reviewed in ref. 1). Protein movement to the nucleus occurs in at least two steps; binding at the cytoplasmic face of the nuclear pore complex is followed by ATP-dependent transport through the nuclear pore channel (2, 3). Proteins that recognize NLSs and that may mediate the binding step have been identified (reviewed in ref. 4). Some proteins of the nuclear pore complex have been shown to be necessary for proper transport (reviewed in refs. 5 and 6).

Less is known about exit of macromolecules from the nucleus. Experiments with microinjected RNAs indicate that exit from the nucleus is an energy-dependent, facilitated process that occurs at the nuclear pore (7). Some mRNAs leave the nucleus in direct association with proteins (8, 9). Some RNA-binding proteins shuttle in and out of the nucleus and have been proposed as candidates for such mRNA carrier proteins (10).

Recent studies have demonstrated a role for a Ras-like GTP-binding protein, Ran/TC4 (11, 12), in nuclear protein import *in vitro*. Experiments using cell-free systems have demonstrated a requirement for soluble cytosolic proteins for proper nuclear protein import (13–16). Ran/TC4 was shown to be a key factor for nuclear protein uptake in semi-intact liver cells (17, 18). Ran/TC4 can be found in both the nucleus and the cytoplasm (19). When in the nucleus it is bound to RCC1, a protein that stimulates guanine nucleotide exchange by Ran/TC4 (20).

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RCC1 has been shown to be important for RNA metabolism and, in particular, for export of RNA from the nucleus (21, 22). Yeast and mammalian cells containing conditional mutations in RCC1 accumulate nuclear poly(A)$^+$ RNA at the nonpermissive temperature, consistent with a block in mRNA export or a step preceding mRNA export, such as release from the RNA processing machinery. RCC1 is primarily nuclear, binds DNA, and has been proposed to participate in regulation of the cell cycle via chromosome condensation (23, 24).

The yeast genes *PRP20*, *GSP1*, and *GSP2* encode homologues of RCC1 and Ran/TC4 (25–27). *GSP1* and *GSP2* were identified by high-copy suppression of *prp20*^{ts} mutants. [The same two genes were also identified by their homology to Ran/TC4 and were independently named *CNR1* and *CNR2* (22). Here the terms *GSP1* and *GSP2* will be used.] *GSP1* is essential for normal cell growth; *GSP2* is dispensable.

In this study, we have tested the role of the yeast Ran/TC4 homologue in transport in and out of the nucleus. We find that the expression in yeast of a form of Gsp1 that can bind but not hydrolyze GTP blocks both protein import into the nucleus and accumulation of poly(A)$^+$ RNA in the cytoplasm. Taken together, these results suggest that GTP hydrolysis by Ran/TC4 is required not only for nuclear import but also for movement of RNA from the nucleus to the cytoplasm.

MATERIALS AND METHODS

Yeast Strains and Plasmids. All experiments were performed with the haploid strain FY86 (*MATα, ura3-52, leu2Δ1, his3Δ200*) (a gift from Fred Winston). Plasmid p_{GAL} contains the *GAL1/10* promoter (28) as an 829-bp fragment plus a multiple cloning site inserted between the *Nru*I and *Hin*dIII sites of YCp50 (*URA3*, *CEN4*). Plasmids p_{GAL}-GSP1 and p_{GAL}-GSP1-G21V contain the PCR-amplified coding sequences of wild-type and mutant (Gly^{21}→Val) *GSP1* (27) as a *Bam*HI–*Eco*RI fragment downstream of the *GAL1* promoter. The sequence was verified by DNA sequencing of the entire coding regions. Another set of plasmids was constructed where *URA3* was replaced by *LEU2*. For construction of plasmids encoding glutathione *S*-transferase (GST)–Gsp1 fusion proteins in *Escherichia coli* (pGEX-GSP1) or yeast (p_{GAL}-GST–GSP1), the *Bam*HI–*Eco*RI fragment was inserted into the pGEX-2T vector (Pharmacia) or into the *GAL1* promoter-containing plasmid pEMBLGST (*URA3*, 2μ) (29). The construction of the plasmids containing the *GAL1* promoter-driven gene fusion of histone H2B1 to the green fluorescent protein (GFP) (ref. 40 and J.D.J.L., unpublished work) and c-Myc epitope-tagged Npl3 (42) will be described elsewhere.

Abbreviations: NLS, nuclear localization sequence; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; GST, glutathione *S*-transferase; GFP, green fluorescent protein.

§To whom reprint requests should be addressed.

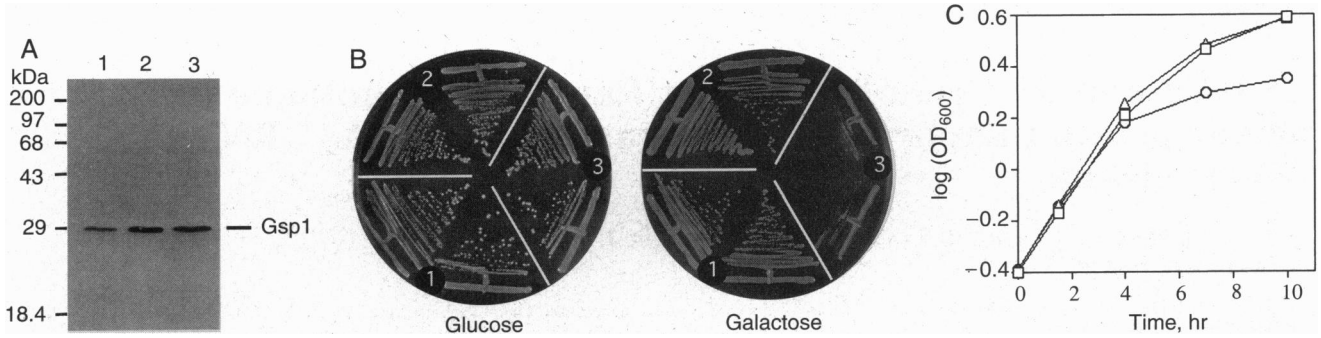

FIG. 1. Expression of Gsp1 and Gsp1-G21V in yeast. (*A*) Cell extracts prepared from yeast strain FY86 bearing a plasmid containing the *GAL1* promoter, p_{GAL} (lane 1), the plasmid p_{GAL}-GSP1 (lane 2), or the plasmid p_{GAL}-GSP1-G21V (lane 3) shifted to galactose for 3 hr were analyzed by SDS/PAGE and immunoblotting with anti-Gsp1 antibodies. (*B*) Cells bearing the plasmid p_{GAL} (sector 1), p_{GAL}-GSP1 (sector 2), or p_{GAL}-GSP1-G21V (sector 3) were streaked on plates missing uracil and containing glucose (*Left*) or galactose (*Right*). (*C*) Cultures of cells containing p_{GAL} (□), p_{GAL}-GSP1 (△), or p_{GAL}-GSP1-G21V (○) were grown in 2% raffinose. The effect of addition of 2% galactose (at 0 hr) on growth rates was determined by measurement of the optical density of the culture.

The *URA3*, *GAL1* promoter plasmid encoding NLS–β-galactosidase was described before (30).

Preparation of Gsp1 Protein and Measurement of GTPase Activity. Yeast cultures of cells bearing plasmid p_{GAL}-GST–GSP1 or p_{GAL}-GST–GSP1-G21V were grown in 2% raffinose to an OD_{600} of 2. Extracts were prepared 2 hr after addition of 2% galactose by glass-bead lysis (16) using buffer 1 [25 mM Tris·HCl, pH 7.5/150 mM potassium acetate/5 mM $MgCl_2$/0.5 mM EGTA/10% (vol/vol) glycerol/0.2 mM dithiothreitol/0.1% (vol/vol) Triton X-100]. Lysates were incubated with glutathione-Sepharose 4B (Pharmacia) for 30 min a 4°C. The beads were washed twice with buffer 1 and twice with buffer 2 (25 mM Tris·HCl, pH 7.5/150 mM potassium acetate/5 mM $MgCl_2$/10% glycerol/0.2 mM dithiothreitol). The bound GST fusion proteins (≈400 μg bound to 1 ml of Sepharose) were loaded with [α-^{32}P]GTP or [γ-^{32}P]GTP (DuPont/NEN, 6000 Ci/mmol; 1 Ci = 37 GBq) at 5 μCi/ml for 20 min at 24°C. The resin was washed three times with buffer 2 and aliquoted on ice. Triplicate samples were incubated with gentle shaking for up to 6 hr at 24°C. After three washes with cold buffer 2, the radioactivity remaining bound to the resin was determined by scintillation counting. GTP hydrolysis rates were calculated after correction for loss of GTP due to exchange and/or dissociation, which is reflected by the loss of bound [α-^{32}P]GTP.

The levels of Gsp1 and the identity of the GST fusion proteins were verified by immunoblotting using antibodies against GST (Santa Cruz Biotechnology, Santa Cruz, CA) and/or Gsp1, with the enhanced chemiluminescence (ECL) detection kit of Amersham. GST–Gsp1 protein was isolated from glutathione-Sepharose according to the instructions from Pharmacia and was > 90% pure.

Protein and RNA Localization. Yeast cell cultures were grown to a density of 10^7 cells per ml at 30°C in media missing uracil and/or leucine with 2% raffinose. Cells were prepared for immunofluorescence (31) at 90 min, 3 hr, or 6 hr after addition of 2% galactose. Incubation with antibodies against Npl3, Myc (mouse monoclonal antibody 9E10), and β-galactosidase was followed by incubation with fluorescein isothiocyanate (FITC)-labeled antibodies (Jackson ImmunoResearch) at a 1:1000 dilution and with 4′,6-diamidino-2-phenylindole (DAPI). Poly(A)$^+$ RNA was localized (32) by *in situ* hybridization of permeabilized fixed cells with a $(dT)_{50}$ probe (end-labeled with digoxigenin-dUTP by terminal deoxynucleotidyltransferase) followed by immunofluorescence with FITC-conjugated anti-digoxigenin Fab fragments. RNase A (0.1 mg/ml, Sigma) was added for 30 min at 37°C in 0.1 M K_2HPO_4.

RESULTS

To test the role of Ran/TC4 in nuclear transport *in vivo*, we constructed yeast strains that overproduce either wild-type Gsp1 or a form of Gsp1 with glycine-21 converted to valine (Gsp1-G21V), both under control of the regulatable *GAL1* promoter. The corresponding mutation in Ran/TC4 is G19V (33). By analogy to both Ras and Ran/TC4, Gsp1-G21V should bind but not hydrolyze GTP, thus stabilizing the GTP-bound form (34, 35). Expression of Gsp1-G21V in

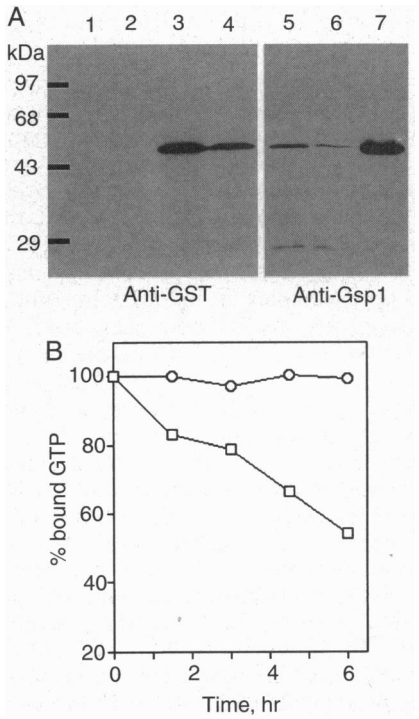

FIG. 2. Expression of GST fused to wild-type and mutant Gsp1 in yeast and assay of GTP hydrolysis. (*A*) Yeast cells bearing the plasmid p_{GAL}-GST–GSP1 (lanes 1, 3, and 5) or p_{GAL}-GST–GSP1-G21V (lanes 2, 4, and 6) were grown in raffinose cultures (lanes 1 and 2) or shifted for 2 hr to 2% galactose (lanes 3–6). Cell extracts (lanes 1–6) or purified GST–Gsp1 fusion protein (lane 7) were analyzed by SDS/PAGE and immunoblotting with mouse monoclonal anti-GST antibodies (lanes 1–4) or rabbit anti-Gsp1 antibodies (lanes 5–7). (*B*) GST fusion proteins with wild-type (□) or mutant (○) Gsp1 were bound to glutathione-Sepharose and tested for GTPase activity as described in *Material and Methods*. The proteins were complexed to [γ-^{32}P]GTP and unbound GTP was removed. The remaining radioactivity bound to protein was determined over the indicated time at 24°C.

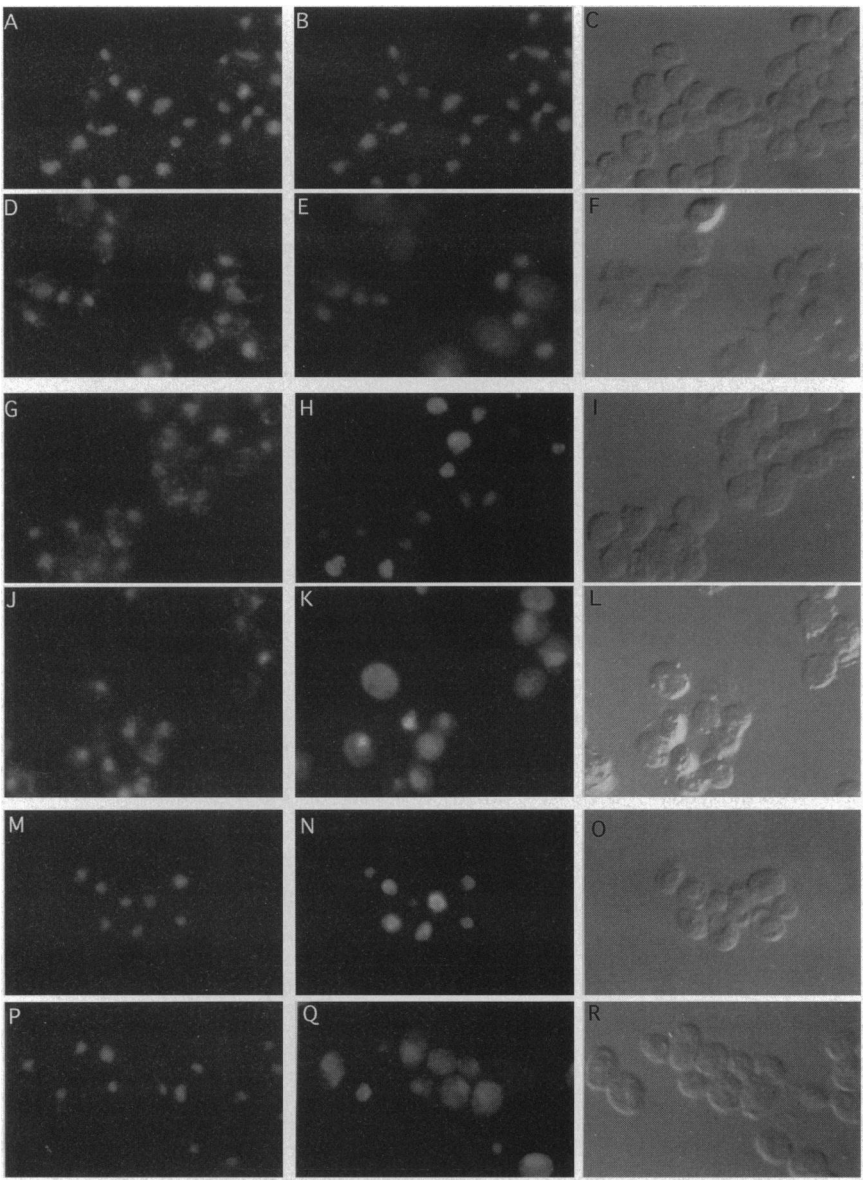

FIG. 3. Localization of nuclear proteins in cells overexpressing Gsp1 or Gsp1–G21V. Yeast cells overproducing Gsp1 (*A–C, G–I,* and *M–O*) or Gsp1–G21V (*D–F, J–L,* and *P–R*) were stained with DAPI (*A, D, G, J, M,* and *P*), viewed with Nomarski optics (*C, F, I, L, O,* and *R*), or stained with anti-Npl3 antibody followed by FITC-labeled anti-rabbit antibody to visualize endogenous Npl3 (*B* and *E*), or with anti-Myc 9E10 monoclonal antibody followed by FITC-labeled anti-mouse antibody to visualize Npl3–Myc (*H* and *K*), or with anti-β-galactosidase antibody followed by FITC-labeled anti-mouse antibody to visualize NLS–β-galactosidase (*N* and *Q*).

otherwise wild-type yeast cells was previously shown to have a dominant lethal effect on cell growth (27).

We estimated the amount of Gsp1 in cells as ≈0.5% of total soluble protein by comparison of the amount of Gsp1 in lysates of wild-type cells to known amounts of isolated GST–Gsp1 (see below) on immunoblots. When plasmid-containing cells were shifted to galactose, wild-type Gsp1 and Gsp1-G21V were produced at levels of about 4-fold and 2-fold higher than endogenous Gsp1 as determined by immunoblot analysis with anti-Gsp1 antibodies (Fig. 1*A*). Cells expressing either Gsp1 or Gsp1-G21V grew normally on glucose medium (where the *GAL1* promoter is repressed). On the other hand, cells producing Gsp1-G21V grew poorly on galactose (Fig. 1*B*). In liquid culture, following a shift to galactose, these cells continued to grow normally for about 4 hr, after which growth began to slow (Fig. 1*C*).

To analyze the ability of Gsp1 to hydrolyze GTP, we constructed plasmids that encoded all of GST fused to all of Gsp1 and Gsp1-G21V at the N terminus and that contained either the strong bacterial *tac* promoter or the yeast *GAL1* promoter. When these plasmid-borne genes were expressed in bacteria, GST–Gsp1 and GST–Gsp1-G21V proteins were produced (data not shown). Unfortunately, neither could be extracted from cell lysates in active form, whereas similar fusion proteins containing GST fused to human Ran/TC4 could (S. Richards and I. Macara, personal communication). On the other hand, both proteins were expressed in a galactose-dependent manner in yeast, were soluble, and could be purified by binding to glutathione-Sepharose (Fig. 2*A*). The ability of GST–Gsp1 and GST–Gsp1-G21V to hydrolyze GTP was tested. After 6 hr at 24°C, wild-type GST–Gsp1 showed about 50% hydrolysis whereas no GTPase activity was observed for the mutant protein (Fig. 2*B*). Similar slow rates of catalysis have been reported for Ran/TC4 (36), and the GTPase activity of Ran/TC4 is also compromised by a similar mutation (S. Richards and I. Macara, personal communication).

Cells producing the Gsp1-G21V mutant do not properly localize nuclear proteins. After a 3-hr shift to galactose, the localization of a normally nuclear RNA-binding protein, Npl3, was unchanged in cells overexpressing wild-type Gsp1; all of the protein was located in the nucleus as determined by immunofluorescence (Fig. 3 *A–C*). In contrast, ≈50% of the cells overexpressing Gsp1-G21V showed Npl3 in both the nucleus and the cytoplasm (Fig. 3 *D–F*). Cytoplasmic localization was observed as soon as 90 min after a shift to galactose.

To examine the affect of Gsp1 on newly synthesized nuclear proteins, we transformed cells with a second plasmid encoding

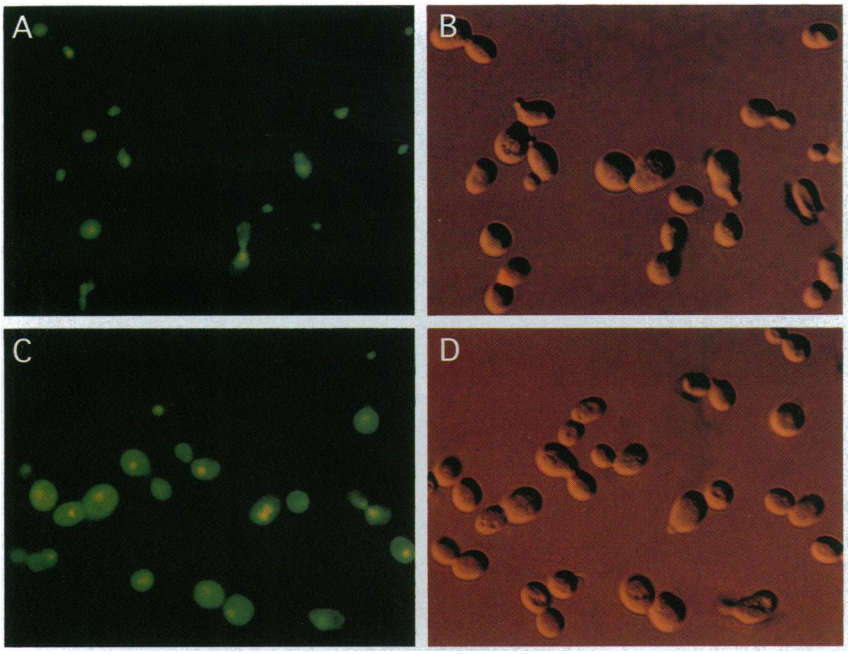

FIG. 4. Cells viewed directly by fluorescence microscopy after a 3-hr shift to galactose to localize H2B1–GFP in living cells. Cells expressed wild-type Gsp1 (*A* and *B*) or Gsp1-G21V (*C* and *D*).

either a Myc epitope-tagged version of Npl3 (Npl3–Myc), or a fusion protein containing the histone H2B NLS fused to β-galactosidase (NLS–β-galactosidase), both also under the control of the inducible *GAL1* promoter. The synthesis of these normally nuclear-targeted reporter proteins was induced at the same time as that of Gsp1 and Gsp1-G21V. After a 3-hr shift to galactose both Npl3–Myc and NLS–β-galactosidase were localized only to the nucleus in cells overexpressing wild-type Gsp1, as determined by immunofluorescence with anti-Myc (Fig. 3 *G–I*) and anti-β-galactosidase antibodies (Fig. 3 *M–O*). In contrast, cytoplasmic Npl3–Myc (Fig. 3 *J–L*) and NLS–β-galactosidase (Fig. 3 *P–R*) accumulated in the cells that simultaneously overexpressed Gsp1-G21V. Mislocalization to the cytoplasm occurred in up to 80% of the cells. Overproduction of other proteins such as Npl3 itself and histone H2B do not disrupt nuclear localization even though their presence at high levels slows cell growth in a manner similar to Gsp1-G21V. Likewise, production of similarly high levels of another GTP-binding protein, Cdc42, and its GTP-bound form had no effect on nuclear protein localization (data not shown).

Mislocalization of nuclear proteins to the cytoplasm was also observed directly in unfixed cells overexpressing Gsp1-G21V. A fusion protein containing all of histone H2B1 fused to the naturally green fluorescent protein (H2B1–GFP) under control of the *GAL1* promoter was made in galactose-grown yeast. Subcellular distribution was determined by direct observation of cells with the fluorescence microscope. Thus, no fixation or antibody treatment was required to localize this protein. After a 3-hr shift to galactose, H2B1–GFP remained in the nucleus in cells overexpressing Gsp1 (Fig. 4 *A* and *B*). However, cytoplasmic H2B1–GFP was observed after 3 hr of galactose-dependent induction of Gsp1-G21V (Fig. 4 *C* and *D*).

Cells producing Gsp1-G21V also did not show proper accumulation of poly(A)$^+$ RNA in the cytoplasm. The distribution of poly(A)$^+$ RNA was determined by *in situ* hybridization with a digoxigenin-labeled $(dT)_{50}$ probe. In cells overexpressing wild-type Gsp1 grown for 3 hr in galactose medium (Fig. 5 *A–C*), poly(A)$^+$ RNA appeared to be distributed throughout the cytoplasm, consistent with normal export of mRNA from the nucleus. In contrast, cells overexpressing Gsp1-G21V showed most of the poly(A)$^+$ RNA concentrated in the nucleus in about 50% of the cells at 3 hr [53 out of a field of 104 cells showed nuclear accumulation of poly(A)$^+$ RNA] (Fig. 5 *G–I*). The same cells grown in glucose medium (and thus not expressing Gsp1-G21V) showed normal cytoplasmic distribution of poly(A)$^+$ RNA (Fig. 5 *D–F*). The hybridization of the probe was dependent on RNA, since treatment with RNase eliminated the signal (Fig. 5 *J–L*).

DISCUSSION

We have shown that when wild-type yeast cells contain the GTP-bound form of the Ran/TC4 homologue Gsp1, movement of macromolecules across the nuclear envelope is disrupted *in vivo*. Proteins that are normally found in the nucleus accumulate in the cytoplasm. Poly(A)$^+$ RNA that is normally cytoplasmic accumulates in the nucleus, suggesting a block in its export from the nucleus.

Experiments with cell-free systems suggested a direct role for Ran/TC4 in nuclear protein import (17, 18). The results presented here provide evidence for a role *in vivo* for Ran/TC4 in movement of macromolecules both into and out of the nucleus. Moreover, our results suggest that GTP hydrolysis by Ran/TC4 is important for these processes to proceed correctly.

How could Ran/TC4 affect traffic in both directions across the nuclear envelope? Once in the nucleus, Ran/TC4 interacts with RCC1 [or the yeast homologue of RCC1 encoded by *PRP20* (26)], which catalyzes GDP–GTP exchange on Ran/TC4 (20). Yeast containing temperature-sensitive mutations in *PRP20* also show defects in mRNA export (21, 22), much like those we have observed for cells bearing Gsp1-G21V. There are several possibilities for how Ran/TC4 could be involved in protein import and RNA export. Ran/TC4 and RCC1 might be mechanistically involved in the processes directly (37), by analogy to the functioning of Rab proteins in vesicle fusion and secretion (41). In this model, proteins and/or RNAs would be targeted to their correct destination in concert with the GTP-bound form of Ran/TC4. Hydrolysis and/or exchange of GDP for GTP would then be required for release of the transported molecule in the correct location. In a second model, blocking uptake of nuclear proteins might result in factors essential for RNA export to accumulate in the cytoplasm, resulting in a concomitant block of appearance of cytoplasmic RNA. A third possibility is that retention of poly(A)$^+$ RNA in the nucleus could be unrelated to transport through the pore *per se*. Instead, the RNA could be trapped in

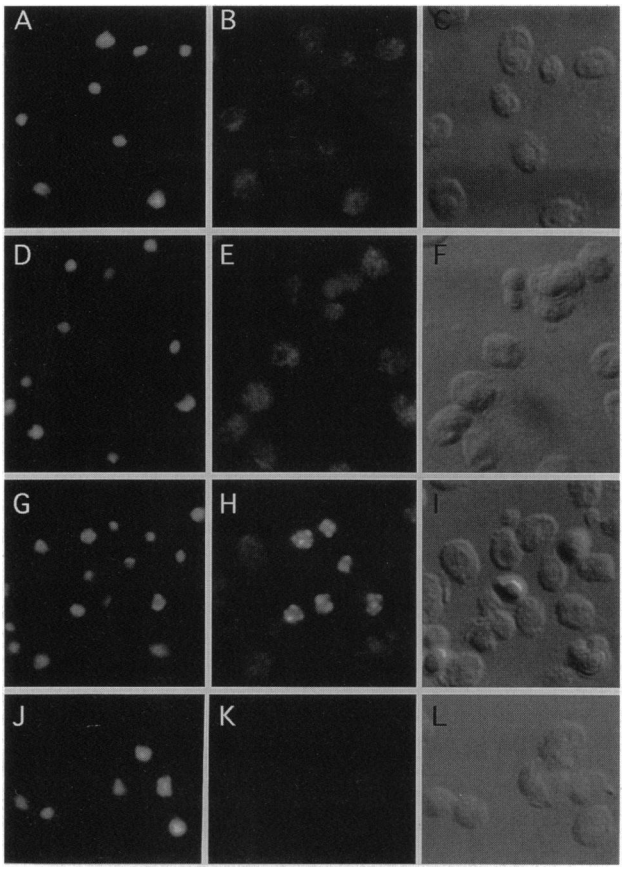

FIG. 5. Localization of poly(A)$^+$ RNA in cells overexpressing Gsp1 or Gsp1-G21V by *in situ* hybridization with oligonucleotide $(dT)_{50}$ probe. Cells were probed with digoxigenin-labeled oligonucleotide $(dT)_{50}$ followed by FITC-conjugated anti-digoxigenin antibody (*B, E, H,* and *K*), or stained with DAPI (*A, D, G,* and *J*) or viewed by Nomarski optics (*C, F, I,* and *L*). (*A–C*) Cells bearing the plasmid p_{GAL}-GSP1, grown for 3 hr in galactose. (*D–I*) Cells bearing the plasmid p_{GAL}-GSP1-G21V, grown for 3 hr in glucose (*D–F*) or galactose (*G–I*). (*J–L*) Cells containing the plasmid p_{GAL}-GSP1-G21V, grown in galactose for 3 hr and probed with oligonucleotide $(dT)_{50}$ and additionally treated with RNase.

condensed chromatin or altered nuclear structures induced by the presence of the mutant form of Gsp1.

Our results do not exclude the possibility that Ran/TC4 and RCC1 also serve to couple nuclear events with the control of cell growth. High levels of transport into and out of the nucleus and other Ran/RCC1 regulated functions, such as chromosome condensation and DNA replication, occur only in dividing cells (38, 39). A central signaling pathway that mediates growth control could have Ran/RCC1 as an intermediate target. The nuclear pore complex could "open" in response to presentation of a NLS-bearing protein but might independently require Ran/TC4 to sense that the cell is active and that protein and RNA transport is appropriate.

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