MOLECULAR AND CELLULAR BIOLOGY, July 1997, p. 3755–3767 Vol. 17, No. 7
0270-7306/97/$04.00+0

Interaction between the Small GTPase Ran/Gsp1p and Ntf2p Is Required for Nuclear Transport

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Received 4 February 1997/Returned for modification 9 April 1997/Accepted 17 April 1997

Bidirectional movement of proteins and RNAs across the nuclear envelope requires Ran, a Ras-like GTPase. A genetic screen of the yeast *Saccharomyces cerevisiae* was performed to isolate conditional alleles of *GSP1*, a gene that encodes a homolog of Ran. Two temperature-sensitive alleles, *gsp1-1* and *gsp1-2*, were isolated. The mutations in these two alleles map to regions that are structurally conserved between different members of the Ras family. Each mutant strain exhibits various nuclear transport defects. Both biochemical and genetic experiments indicate a decreased interaction between Ntf2p, a factor which is required for protein import, and the mutant *GSP1* gene products. Overexpression of *NTF2* can suppress the temperature sensitive phenotype of *gsp1-1* and *gsp1-2* and partially rescue nuclear transport defects. However, overexpression of a mutant allele of *NTF2* with decreased binding to Gsp1p cannot rescue the temperature sensitivity of *gsp1-1* and *gsp1-2*. Taken together, these data demonstrate that the interaction between Gsp1p and Ntf2p is critical for nuclear transport.

A common theme in the regulation of biological processes is the involvement of GTP-binding proteins of the Ras superfamily (16). These small GTPases cycle between GTP- and GDP-bound states. They can function as molecular "on/off" switches as in Ras (16), or they can be more like the Rab proteins involved in vesicular transport (63). In this case, the GTP- and GDP-bound states of Rab are involved in the vectorial transport of vesicles to their target membrane. Movement of macromolecules in and out of the nucleus is a highly regulated process which is also controlled by a Ras-like GTPase termed Ran (24, 46, 52). This process of nuclear transport occurs via large proteinaceous channels termed nuclear pores which are embedded in the nuclear envelope.

Many of the proteins destined for the nucleus contain short stretches of basic amino acids termed nuclear localization sequences (NLSs), which are necessary for proper import into the nucleus (34, 38, 75). Examples of the classical NLS include those found in SV40, yeast histone 2B protein, and the bipartite NLS in nucleoplasmin. A soluble receptor found in the cytoplasm recognizes NLSs and targets them to the nuclear pore. The NLS receptor is composed of two subunits. An ~60-kDa protein called p54/56 (2), Srp1p (79), importin-α (39), or karyopherin α (54) recognizes the NLS. The second subunit, termed p97 (1, 19), importin 90/β (29, 40), or karyopherin β (65), is required for targeting the NLS-bearing protein to the nuclear pore.

There are other proteins that do not contain a classical NLS but are nevertheless efficiently transported into the nucleus. One such example is the heteronuclear RNA binding proteins (hnRNPs). In mammalian cells, the protein hnRNP A1 can rapidly shuttle in and out of the nucleus yet it does not contain a classical NLS. Instead, it contains a bifunctional import-export sequence known as an M9 sequence (47, 76). Import of hnRNP A1 uses a mechanism independent of importin-α–importin-β. An importin-β-like molecule termed transportin appears to mediate import. Transportin-mediated import of hnRNP A1 does not involve importin-α (64).

In addition to these receptor molecules, the nuclear transport machinery includes several other factors. Like that of Ras, the GTPase activity of Ran is negligible until it is activated by a GTPase-activating protein (GAP). Rna1p (36) is the identified GAP in yeast (7, 22). In vertebrates, it is known as RanGAP1 (9, 10). Once in the GDP-bound state, Ran must be recycled to the GTP-bound state. An exchange factor, Prp20p (3) (RCC1 in higher eukaryotes), accomplishes this task (12, 56). Two additional proteins interact with Ran to promote translocation across the nuclear pore. Yrb1p (RanBP1 in higher eukaryotes) is required for protein import and poly(A)$^+$ RNA export (18, 72). It interacts with Ran in the GTP-bound state and further accelerates GTP hydrolysis in the presence of Rna1p (9, 11, 72). The other protein is Ntf2p (also known as p10 or pp15 [23, 51, 61]). Ntf2p can be found as a dimer in a complex with Ran (51, 55, 61).

The precise role for Ntf2p has not been established although it has been suggested that it functions to coordinate the association-dissociation of an import complex at the nuclear pore (55). However, there is currently controversy over the importance of Ntf2p in nuclear transport. In some in vitro nuclear import assays, the addition of Ntf2p appears to be unnecessary to promote import (18, 30, 31), whereas in others it facilitates transport (51, 61). Furthermore, conditional alleles of yeast *NTF2* exhibit protein import defects in vivo (23).

In the yeast *Saccharomyces cerevisiae*, there are two *ran* homologs, *GSP1* and *GSP2* or *CNR1* and *CNR2. GSP1* was originally isolated as a high-copy suppressor of *prp20-1*, a mutant allele of the exchange factor (8). A second gene, *GSP2*, whose gene product is greater than 95% identical to Gsp1p, was also identified in the same screen. *CNR1* and *CNR2* were isolated in a similar screen and are identical to *GSP1* and

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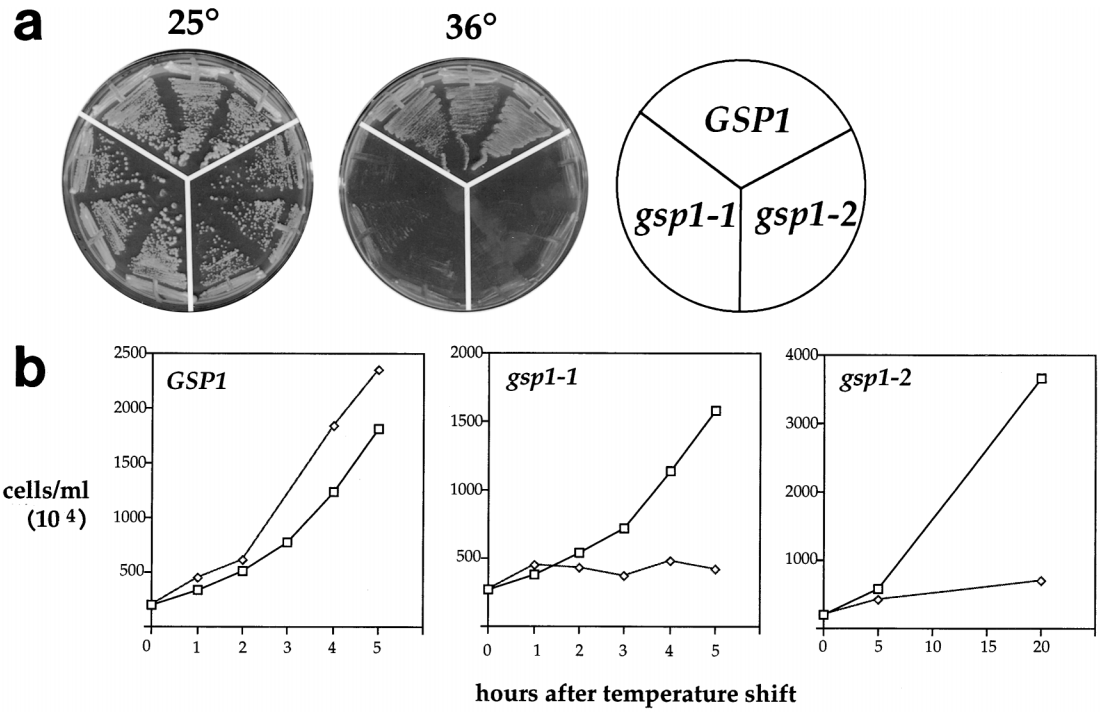

FIG. 1. Characterization of *gsp1-1* and *gsp1-2* mutant phenotypes. (a) *GSP1*, *gsp1-1*, and *gsp1-2* strains were streaked to YEPD plates and incubated at 25°C or 36°C for 2 days. (b) Growth curves for *GSP1*, *gsp1-1*, and *gsp1-2* strains at 25°C (□) and 36°C (◇). Overnight cultures of *GSP1*, *gsp1-1*, and *gsp1-2* strains were diluted into YEPD medium and grown for 4 h at room temperature. Half of each culture was shifted to 36°C. Aliquots of cells were removed at the indicated times and counted.

GSP2 (37). Both gene products are approximately 90% similar to vertebrate Ran, indicating they are the yeast Ran homologs (8). Of the two genes, only *GSP1* is essential. Although the gene products of *GSP1* and *GSP2* are virtually identical, *GSP2* can only rescue the *GSP1* null strain if it is present on a high-copy plasmid (8). The physiological role of *GSP2* is unclear, but it may be required only under certain growth conditions.

In order to understand how Ran regulates nuclear transport, we have used the yeast *S. cerevisiae* as a model system. We have generated two conditional alleles of *GSP1* and studied the effects of the mutations both biochemically and genetically to determine the molecular basis for the loss of function of Gsp1p. From this analysis, we demonstrate that Gsp1p is required for both classical and nonclassical nuclear import pathways and that an interaction between Gsp1p and Ntf2p is required for efficient nuclear transport.

MATERIALS AND METHODS

All chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) except where noted. All DNA manipulations were performed according to standard methods (69), and all yeast media were prepared by standard procedures (66).

Plasmids. The plasmid YEp24-*GSP1* (pPS984) was a gift of M. Clark (University of Toronto). Plasmid pPS985 was generated by cloning an ~2.8-kb *Hin*cII/*Pst*I fragment from YEp24-*GSP1* into pBluescript (Stratagene, La Jolla, Calif.). Plasmid pPS965 was made by cloning an ~3-kb *Apa*I/*Sac*I fragment from pPS985 into pRS314 (*CEN-TRP1*) (74). Plasmids pPS986 (*CEN-TRP1-gsp1-1*) and pPS987 (*CEN-TRP1-gsp1-2*) are the original plasmids isolated from the genetic screen described below.

The plasmids pPS1023 (2μm *NTF2*), pPS1024 (2μm *ntf2-1*) and pPS1025 (2μm *ntf2-2*) were made by cloning a 3-kb *Pst*I/*Sac*I fragment from pPS882 (*CEN-LEU2-NTF2*), pPS919 (*CEN-LEU2-ntf2-1*), and pPS920 (*CEN-LEU2-ntf2-2*) into pRS425 (2μm *LEU2*) (74).

The Ntf2p bacterial expression plasmid pPS982 was made by amplifying the coding region of Ntf2p by PCR with Vent polymerase (New England Biolabs, Beverly, Mass.). Primers were designed to introduce *Bam*HI and *Hin*dIII sites at the 5′ and 3′ ends, respectively. The PCR product was cloned into the T7-based expression vector pMW172 (78).

Two-hybrid constructs were made by amplifying the coding region of wild-type and mutant Ntf2p, Gsp1p, Ran, and Ras by PCR. Primers were designed to introduce various sites at the 5′ and 3′ ends and were then cloned into either pEG202 or pJG4-5 (33). The following sites were added to the various PCR products: *Xho*I and *Xho*I to *GSP1* and *Eco*RI and *Xho*I to *NTF2*, *ran*, and *ras*.

Strains. The coding regions of *GSP1* and *GSP2* were replaced with *HIS3* by a PCR-based knockout protocol (6). PCR with Vent polymerase was used to generate a *HIS3* fragment flanked by 45 bp directly 5′ and 3′ of the *GSP1* and *GSP2* coding regions. The two PCR products were individually transformed into the diploid yeast strain PSY845 (*MAT***a**/α *ura3-52*/*ura3-52 leu2Δ1*/*leu2Δ1 his3Δ200*/*his3Δ200 trp1Δ63*/*TRP1*), and *His*$^+$ transformants were selected. Correct integration of the *HIS3* gene was confirmed by PCR analysis and by Southern blotting. Diploids containing the desired knockout were sporulated, and tetrads were dissected. In the case of the *GSP1* knockout, the diploid strain was first transformed with YEp24-*GSP1* before sporulation and dissection. All complete tetrads showed 2:2 segregation of the *His*$^+$ prototrophy, and for *GSP1* knockouts, all *His*$^+$ spores were sensitive to 5′ fluoroorotic acid indicating that these spores required YEp24-*GSP1* to survive. Yeast strains PSY960 (*MAT*α *gsp2::HIS3 ura3-52 leu2Δ1 his3Δ200*) and PSY961 (*MAT***a** *gsp1::HIS3 ura3-52 leu2Δ1 his3Δ200 trp1Δ63*) were isolated from these dissections. Yeast strain PSY962 (*MAT*α *gsp1::HIS3 gsp2::HIS3 ura3-52 leu2Δ1 his3Δ200 trp1Δ63*) was isolated as one of two *His*$^+$ spores from the nonparental ditype tetrad of a cross between PSY960 and PSY961.

GSP1, *gsp1-1*, and *gsp1-2* strains in this study were made by the plasmid shuffle method (73) where the wild-type plasmid copy of *GSP1* in strain PSY962 was replaced by centromeric plasmids carrying *GSP1*, *gsp1-1*, or *gsp1-2* (pPS965, pPS986, and pPS987, respectively).

PCR mutagenesis. Recessive, temperature-sensitive alleles of *GSP1* were generated by taking advantage of the high error rate of *Taq* polymerase (Promega, Madison, Wis.). An ~1.3-kb PCR product was generated. This product contains the entire *GSP1* open reading frame including 155 bp upstream of the initiation codon and 502 bp downstream of the stop codon. The PCR product was generated with *Taq* polymerase under mutagenic conditions (4 mM $MgCl_2$) and purified on a Qiaquick PCR column (Qiagen, Chatsworth, Calif.). The plasmid pPS965 was linearized with *Cla*I and *Nco*I to remove an ~1.1-kb insert containing the coding region of *GSP1*. Gap repair ligation (57) was achieved by cotransforming the strain PSY962 with the PCR product and linear pPS965. We chose to use the strain PSY962 (Δ*gsp1*Δ*gsp2*) to prevent upregulation of *GSP2* that might rescue any plasmid-linked temperature-sensitive phenotype. Colonies were selected on synthetic complete medium (SC) lacking tryptophan. The plates were then replica plated to SC plates lacking tryptophan, supplemented with 1 mg of 5′ fluoroorotic acid/ml and 7.5 mg of the vital dye Erythrocin B/liter, and placed at 25 and 36°C. Uptake of the Erythrocin B dye by dead cells, which

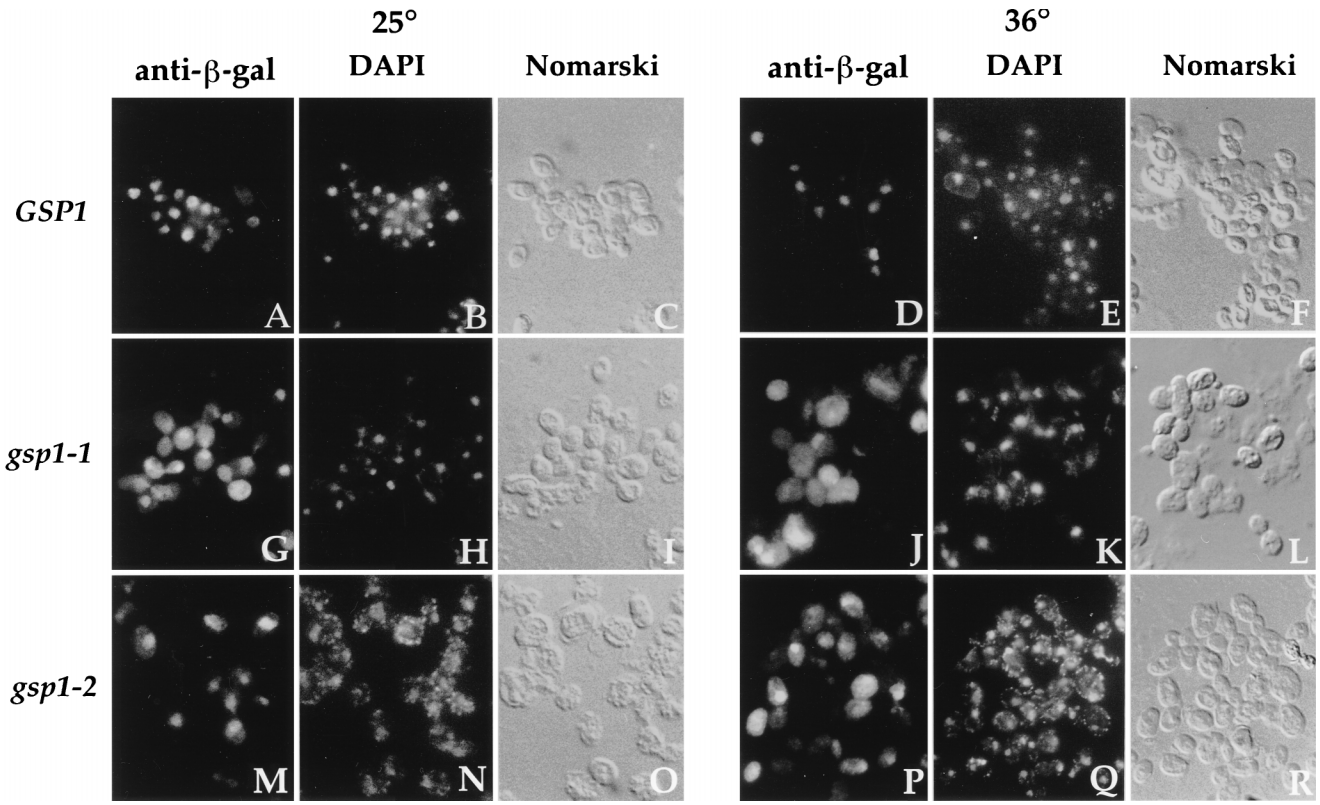

FIG. 2. H2B-NLS-β-galactosidase is mislocalized at the nonpermissive temperature. *GSP1*, *gsp1-1*, and *gsp1-2* strains were transformed with a plasmid encoding the NLS of yeast H2B fused to β-galactosidase (pPS613) (53). Cells were induced for 1 h with 2% galactose, and half the cells were shifted to 36°C for 1 h before being prepared for indirect immunofluorescence microscopy. The reporter construct was visualized with a monoclonal antibody against β-galactosidase (diluted 1:800) (mAb J2F7) at 25°C (A, G, and M) and 36°C (D, J, and P). The nuclei, visualized with DAPI (B, E, H, K, N and Q) and Nomarski optics (C, F, I, L, O and R), correspond to those shown in panels A, D, G, J, M, and P, respectively.

results in a pink colony, was used to identify temperature-sensitive colonies. Plasmids were rescued from temperature-sensitive colonies and retransformed into PSY962 and PSY961 to confirm plasmid linkage of the temperature-sensitive phenotype. Plasmids that retested for temperature sensitivity were sequenced to identify the mutations.

Indirect immunofluorescence microscopy and in situ hybridization. Five milliliters of cells was grown in the appropriate medium to approximately 10^7 cells/ml and fixed with 700 μl of 37% formaldehyde for 60 to 90 min. Following fixation, cells were harvested by centrifugation and washed with 0.1 M potassium phosphate buffer (pH 6.5) and resuspended in 500 μl of P solution (1.2 M sorbitol, 0.1 M potassium phosphate buffer [pH 6.5]). Dithiothreitol (DTT) was added to 25 mM, and the cells were incubated at 30°C for 10 min with gentle agitation followed by addition of 300 μg of Zymolyase 100T (ICN Pharmaceuticals, Costa Mesa, Calif.)/ml (diluted from 10-mg/ml stock in P solution). Digestion of the cell wall was monitored by bright-field microscopy. Digested cells were resuspended in 500 μl of P solution and applied to Teflon-faced microscope slides precoated with 0.3% polylysine. Cells were then permeabilized with 0.5% Triton X-100 in P solution for 5 min. Permeabilized cells were processed for indirect immunofluorescence microscopy or in situ hybridization.

(i) Indirect immunofluorescence microscopy. This procedure is a modification of that described by Flach et al. (26). Briefly, permeabilized cells were treated with blocking solution (5% heat-inactivated fetal calf serum, 0.3% Triton X-100, 0.1 M Tris [pH 9.5], 0.1 M NaCl) for 15 min. Antibodies diluted in blocking solution were applied to cells and incubated for 2 h at room temperature or overnight at 4°C. Cells were washed twice for 10 to 30 min each time with antibody wash 1 (0.1 M Tris [pH 9.0], 0.15 M NaCl). Fluorescein isothiocyanate (FITC)- or Texas Red-coupled secondary antibody (goat anti-rabbit or anti-mouse immunoglobulin G; Jackson Immunologicals, West Grove, Pa.) was diluted 1:1,000 in blocking solution and incubated for 2 h at room temperature. Cells were washed twice with antibody wash 1 for 10 to 30 min and then twice for 10 to 30 min with antibody wash 2 (0.1 M Tris [pH 9.5], 0.1 M NaCl, 50 mM $MgCl_2$). DNA in the nuclei was visualized by treating cells with 1 μg of 4′,6-diamidino-2-phenylindole (DAPI)/ml in antibody wash 2 for 5 min followed by two 5-min rinses with antibody wash 2. Cells were air dried and mounted in antifade solution (1 mg of *p*-phenylene diamine/ml in phosphate-buffered saline [PBS]–90% glycerol) under a coverslip. Slides were sealed with nail polish.

For experiments with the artificial reporter construct H2B-NLS-β-galactosidase, *GSP1*, *gsp1-1*, and *gsp1-2* cells were transformed with pPS613 (53). Single colonies were grown overnight at room temperature in SC medium which lacked uracil and which was supplemented with 2% glucose. The overnight cultures of *GSP1* and *gsp1-1* cells were diluted 1:200 into uracil-lacking SC medium supplemented with 2% raffinose. Cells from *gsp1-2* cultures were collected by centrifugation and resuspended in uracil-lacking SC medium supplemented with 2% raffinose. All raffinose cultures were then grown for 22 h at room temperature followed by a 1-h induction with 2% galactose at room temperature. Half of each culture was shifted to 36°C for 1 h and fixed for 1 h with formaldehyde. Cell walls were digested as described above. Cells were affixed to polylysine-coated slides and permeabilized in ice-cold methanol for 6 min followed by immersion in ice-cold acetone for 30 s. Slides were air dried and processed for indirect immunofluorescence microscopy as described above.

(ii) In situ hybridization. This procedure is a modification of that described by Amberg et al. (5). Triton X-100-permeabilized cells were treated for 2 min with freshly prepared 0.1 M triethanolamine (pH 8.0) followed by a 10-min treatment with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) to block polar groups. The cells were then treated with prehybridization solution (50% deionized formamide, 4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] 1× Denhardt's solution, 125 μg of tRNA/ml, 10% dextran sulfate, 500 μg of denatured salmon sperm DNA/ml) for >1 h at 37°C. Digoxigenin-labeled oligo(dT) probe (50-mer) (prepared with a Genius-6 kit [Boehringer Mannheim, Indianapolis, Ind.]) diluted in prehybridization solution was applied to samples, and samples were incubated overnight at 37°C in a humidified chamber. The following day, cells were washed as follows: 1 h in 2× SSC at room temperature, 1 h in 1× SSC at room temperature, 30 min in 0.5× SSC at 37°C, and 30 min in 0.5× SSC at room temperature. Following hybridization and washes, cells were treated as described above for the indirect immunofluorescence microscopy samples. The hybridized oligonucleotide was visualized with FITC-labeled anti-digoxigenin Fab fragments (Boehringer Mannheim) diluted 1:200 in antibody blocking solution.

Ntf2p binding experiments. *GSP1*, *gsp1-1*, or *gsp1-2* cells were grown overnight at room temperature in yeast extract-peptone-dextrose (YEPD) medium to confluency. Cells were harvested by centrifugation and washed twice with water and once in PBSM (PBS, 3 mM KCl, 2.5 mM $MgCl_2$). Cells were then resus-

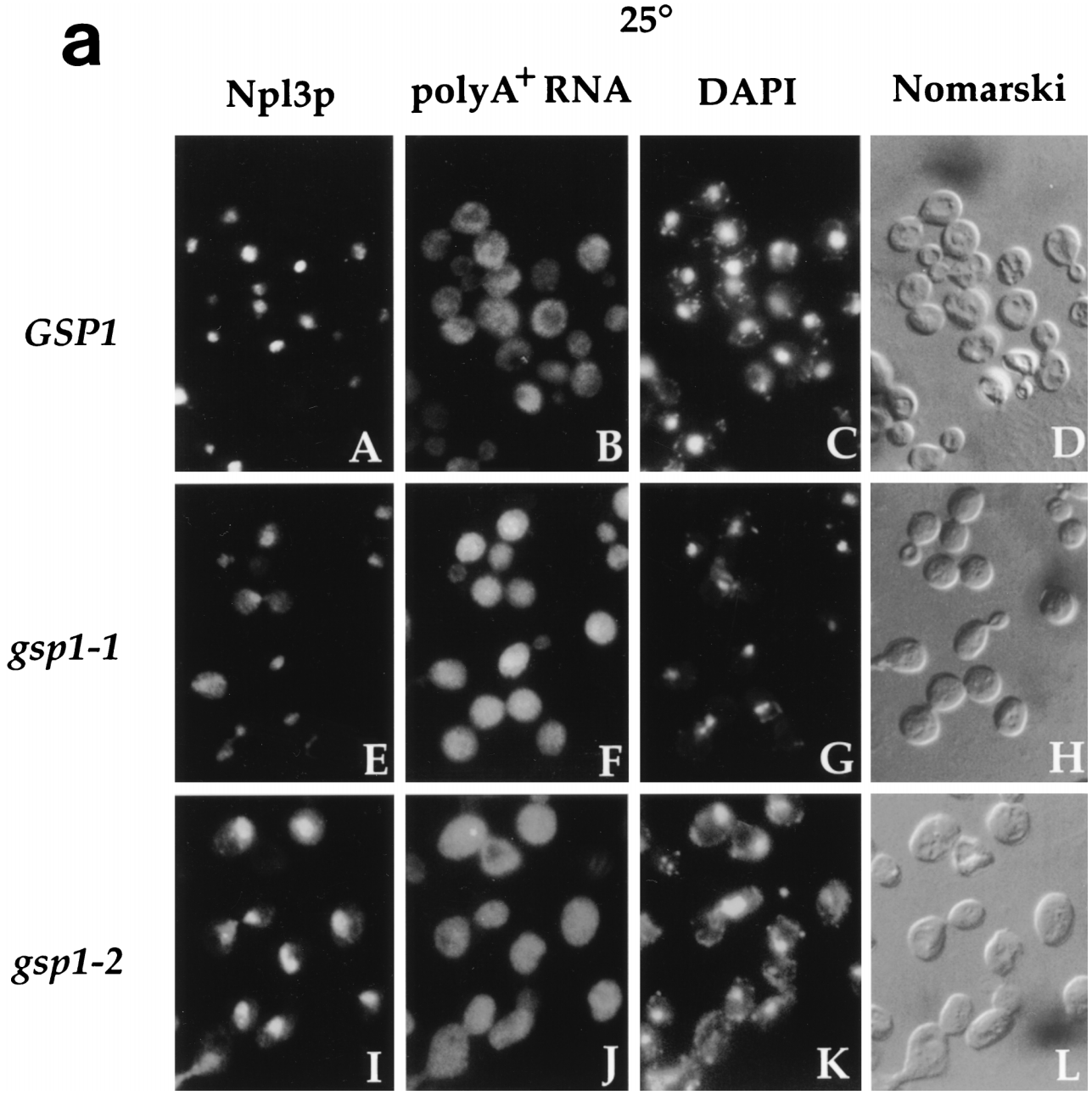

FIG. 3. Conditional *GSP1* alleles show differing levels of transport defects. *GSP1*, *gsp1-1*, and *gsp1-2* strains were grown to approximately 10^7 cells/ml in liquid YEPD medium. Half of the cells were left at room temperature (a) and half were shifted to 36°C for 3 (b). Cells were fixed and processed for both protein import (panels A, E, and I) and poly(A)$^+$ RNA export (panels B, F, and J) assays using indirect immunofluorescence microscopy with an antibody against Npl3p and in situ hybridization with an digoxigenin-labeled dT_{50} oligonucleotide probe, respectively. Npl3p antibodies were visualized with Texas Red–goat anti-rabbit immunoglobulin G, and hybridized nucleotide probe was visualized with FITC-conjugated anti digoxigenin Fab fragments. The corresponding DAPI images (panels C, G, and K) and Nomarski images (panels D, H, and L) are included.

pended in 1 volume of PBSMT (PBS, 3 mM KCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100) supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride [PMSF] and 3 μg each of aprotinin, leupeptin, chymostatin, and pepstatin/ml). One volume of glass beads was added, and cells were lysed with two 60-s pulses in a beadbeater. The resulting lysate was clarified by centrifugation and assayed for protein concentration by using the BioRad Protein Assay Kit (Richmond, Calif.).

Typically, 300 μl of yeast lysate (30 mg/ml) was incubated with 50 μl of Ntf2p-Sepharose beads (see below). Binding was carried out in PBSM (total volume, 1 ml) at 4°C for 1 h. Beads were then washed three times in 1 ml of PBSM. Bound proteins were eluted with 50 μl of sample buffer and resolved by polyacrylamide gel electrophoresis (42) and transferred to nitrocellulose for immunoblotting (77).

Ntf2p purification and immobilization. Yeast Ntf2p was purified from *Escherichia coli* as previously described for rat Ntf2p (39). pPS982 was transformed into *E. coli* BL21(DE3). Transformants were inoculated into 2× tryptone-yeast extract medium containing 100 μg of ampicillin/ml and grown overnight at 34°C. It was not necessary to induce expression as the basal level of expression of the T7 polymerase yielded a large amount of Ntf2p. Bacteria were harvested by centrifugation and stored at −20°C until required.

Ntf2p was isolated by thawing the cell pellets and resuspending them in 25% sucrose–50 mM Tris-HCl [pH 8.0]–5 mM $MgCl_2$–1 mM EGTA–0.1 mM PMSF.

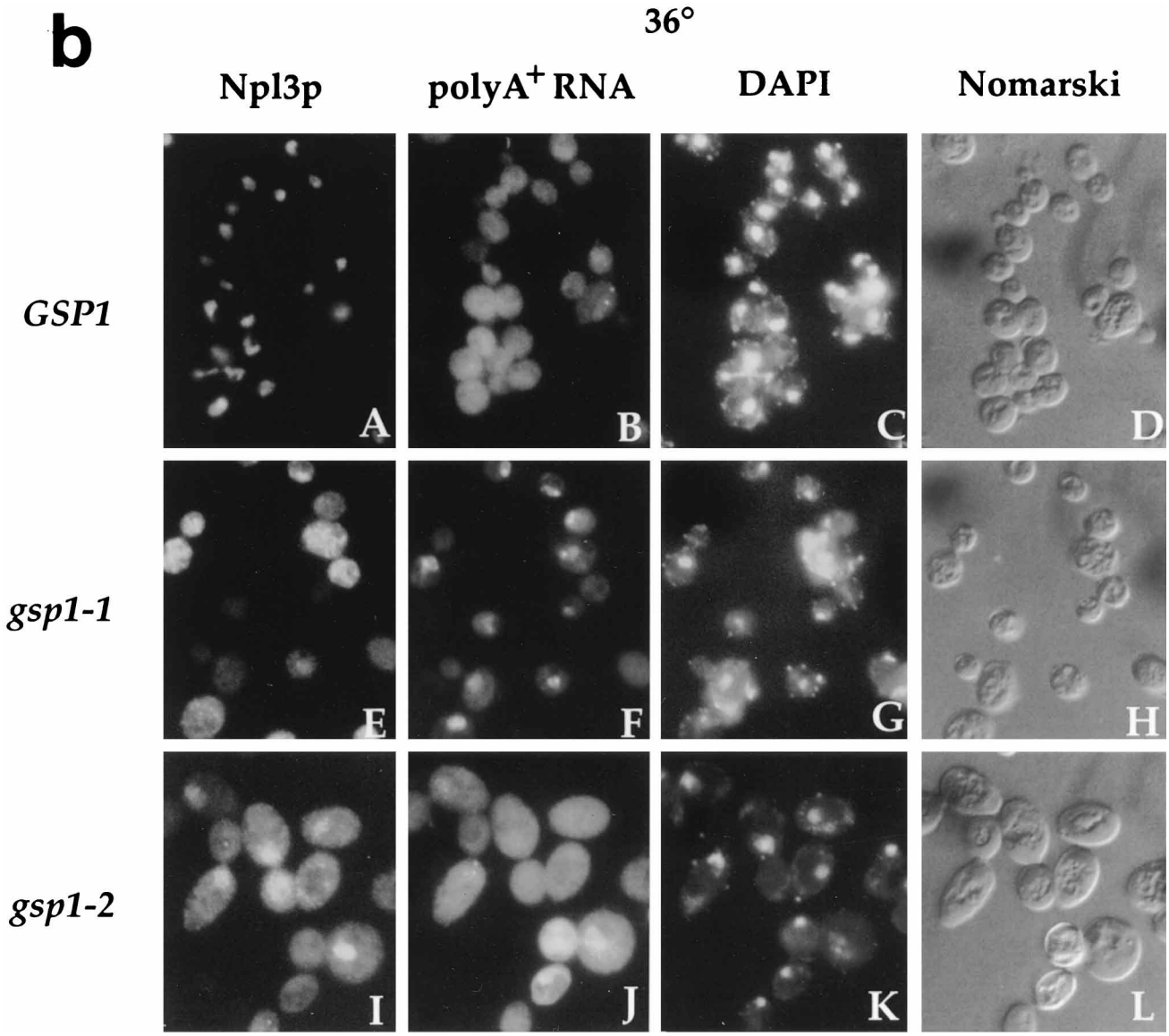

FIG. 3—*Continued.*

Cells were lysed by incubation with 10 mg of lysozyme/ml followed by treatment with DNase. The soluble fraction was isolated by centrifugation at 40,000 × *g* for 15 min and dialyzed overnight against 20 mM Tris-HCl [pH 8.0]–2 mM $MgCl_2$–1 mM DTT–0.1 mM PMSF (NTF2 buffer A). The dialysate was clarified at 40,000 × *g* for 20 min at 4°C and applied to a DE52 ion exchange column (10 by 2.5 cm) and washed with NTF2 buffer A. Ntf2p was eluted with a gradient of 0 to 400 mM NaCl. Fractions containing Ntf2p were pooled, resuspended in a minimal volume of 20 mM Tris-HCl [pH 7.4]–50 mM NaCl–2 mM $MgCl_2$–1 mM DTT–0.1 mM PMSF (NTF2 buffer B), and applied to a column of Sephacryl SR100 (95 by 2.5 cm) preequilibrated in NTF2 buffer B. Fractions containing Ntf2p were collected and pooled.

Purified Ntf2p was cross-linked to CNBr-Sepharose as previously described (21). Briefly, CNBr-Sepharose beads (Pharmacia, Piscataway, N.J.) were swollen and washed in 1 mM HCl. Beads were transferred to coupling buffer (100 mM $NaHCO_3$ [pH 8.3], 500 mM NaCl) and added to 10 mg of Ntf2p in coupling buffer. Coupling was carried out at room temperature for 2 h. Residual active groups were blocked with 1 M Tris-HCl, pH 8.0, for 2 h at room temperature. Beads were then washed successively and extensively four times in coupling buffer and acid wash buffer (0.1 M sodium acetate [pH 4.0], 500 mM NaCl).

Two-hybrid interaction assays. All constructs were tested for expression of a fusion protein of the appropriate size. pEG202 constructs were transformed into EGY48 (*MAT*α *ura3 trp 1 his3 lexAopLEU2*) and pJG4-5 constructs were transformed into EGY42 (*MAT***a** *ura3 trp 1 his3 lexAopLEU2*) (gift of Roger Brent, Massachusetts General Hospital, Boston, Ma.). Strains were crossed, and diploids were selected. An interaction is scored positive if the diploid becomes Leu^+, turns blue on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) indicator plates, and tests positive in β-galactosidase activity assays as described in references 25 and 27. In typical β-galactosidase assays, positive interactions were scored if the optical density at 420 nm (OD_{420}) was between 0.2 and 0.4. For negative interactions, the OD_{420} was <0.05.

RESULTS

Isolation of conditional alleles of *GSP1*. To study the function of Gsp1p in vivo, we generated conditional alleles of *GSP1* and characterized their temperature-sensitive phenotypes. Conditional alleles of *GSP1* were generated in a haploid yeast strain in which the chromosomal copy of *GSP1* was replaced by *HIS3*. Viability of the strain was maintained by a wild-type copy of *GSP1* carried on an episomal plasmid. Since the *GSP2* gene product is virtually identical to that of *GSP1* (8), the chromosomal copy of *GSP2* (which is not essential) was also replaced by *HIS3* to eliminate the possibility that upregulation of *GSP2*

might suppress the *GSP1* temperature-sensitive phenotype. In the screen, the wild-type plasmid copy of *GSP1* was replaced by a PCR-mutagenized copy carried on a centromeric plasmid (see Materials and Methods). After screening approximately 60,000 colonies, two transformants that displayed plasmid-linked temperature sensitivity were found: *gsp1-1* and *gsp1-2* (Fig. 1A). Both strains also exhibited slowed growth in liquid culture compared to cells containing wild-type *GSP1* at the nonpermissive temperature (Fig. 1B).

Mutant alleles of *GSP1* have nuclear transport defects. To determine whether *gsp1-1* and *gsp1-2* strains have nuclear transport defects, we used indirect immunofluorescence microscopy to examine protein import. To assay for nuclear import, we localized an artificial reporter construct consisting of a classical NLS (yeast histone 2B) fused to β-galactosidase (H2B-β-gal) (53) as well as an endogenous protein, Npl3p (15, 26), which does not contain a classical NLS. We chose to localize both classes of nuclear proteins in mutant cells because there is emerging evidence that the import of different proteins occurs via distinct mechanisms (see Discussion).

In cells containing a wild-type copy of *GSP1*, H2B-β-gal was localized to the nucleus at both 25 and 36°C. (Fig. 2A and D). Similarly, in both mutant strains the reporter protein can be detected in the nucleus at the permissive temperature, although a large proportion is cytoplasmic (Fig. 2G and M). Following a 1-h shift to 36°C, H2B-β-gal becomes mislocalized to the cytoplasm in both *gsp1-1* and *gsp1-2* cells (Fig. 2J and P). The nuclear signal observed in some cells at the nonpermissive temperature is most likely due to protein which has been imported into the nucleus prior to the temperature shift.

In cells containing a wild-type copy of *GSP1*, the steady-state distribution of endogenous Npl3p is nuclear at both 25 and 36°C (Fig. 3a and b, panel A). In both mutant strains, Npl3p is mainly nuclear at the permissive temperature (Fig. 3a, panels E and I). Following a 3-h shift to 36°C, Npl3p becomes severely mislocalized to the cytoplasm in *gsp1-1* cells (Fig. 3b, panel E). In *gsp1-2* cells, the majority of Npl3p is cytoplasmic although some nuclear Npl3p is still visible in some cells (Fig. 3b, panel I). The effect of the temperature shift is rapid; Npl3p accumulates in the cytoplasm as early as 10 min after the shift to the nonpermissive temperature in *gsp1-1* cells and after approximately 30 min in *gsp1-2* cells (data not shown). These results demonstrate that protein import into the nucleus is impaired in the absence of a functional copy of *GSP1.*

Poly(A)$^+$ RNA export in these mutants was also examined by using an in situ hybridization assay (see Materials and Methods). In wild-type cells, poly(A)$^+$ RNA is efficiently exported out of the nucleus and is localized in the cytoplasm at 25 and 36°C (Fig. 3a and b, panel B). Both mutant strains also display a cytoplasmic distribution of poly(A)$^+$ RNA at the permissive temperature (Fig. 3a, panels F and J). However, upon shift to 36°C, *gsp1-1* displays a poly(A)$^+$ RNA export defect (Fig. 3b, panel F). In contrast, poly(A)$^+$ RNA export appears unaffected in *gsp1-2* (compare Fig. 3b, panels F and J). The onset of the poly(A)$^+$ RNA export defect can be detected within 30 min of shifting *gsp1-1* cells to 36°C (data not shown).

Mutations in *gsp1-1* and *gsp1-2* map to regions structurally conserved between Ran and Ras. X-ray crystallography studies (71) have shown that although the amino acid sequences of human Ran and Ras are only about 25% homologous (24), the three-dimensional structures of the core domains of the two proteins are remarkably similar. Both structures consist of a G domain constructed from a six-stranded β sheet surrounded by five α helices that is basically conserved between Ras, EF-Tu, transducin-α, and EF-G (71). Ran is, however, significantly different at its C terminus and in the effector loop region. In

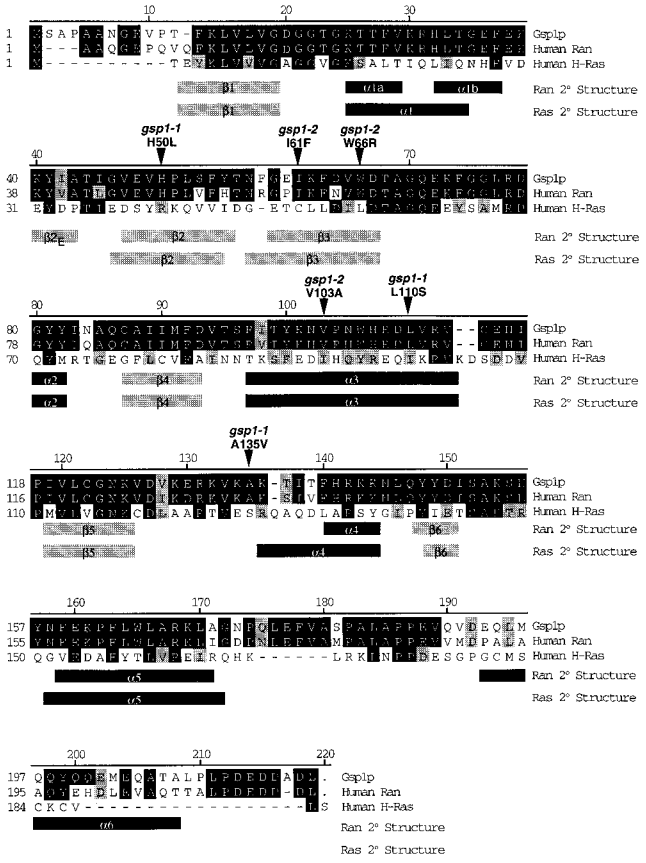

FIG. 4. Sequence and structure comparison between Gsp1p, Ran, and Ras. The protein sequences of Gsp1p, Ran, and human H-Ras were aligned by using the program Megalign (DNAStar). Residues in black indicate identical amino acids while shaded residues indicate similarity with Gsp1p. Sequence numbering follows that of Gsp1p. Secondary structural elements are displayed below the sequence. Shaded boxes indicate β sheets and black boxes indicate α helices. Numbering of the structural elements follows that indicated in Scheffzek et al. (71). The amino acid changes in Gsp1-1p are H50→L (CAT to CTT), L110→S (TTA to TCA) and A135→V (GCC to GTC) and the amino acid changes in Gsp1-2p are in I61→F (ATT to TTT), W66→R (TGG to AGG) and V103→A (GTT to GCT).

Ras, it is the effector loop that binds to several different proteins. Human Ran and Gsp1p are highly homologous (greater than 90%) over most of their amino acid sequences (Fig. 4). Although it has not yet been determined by X-ray crystallography, the three-dimensional structure of Gsp1p is unlikely to differ markedly from that of human Ran. Both mutant alleles of *GSP1* have point mutations that are located in regions that are structurally conserved between human Ran and Ras (see Fig. 4). Each mutant allele of *GSP1* has three point mutations: in the *gsp1-1* gene product these changes are H50→L, L110→S, and A135→V, whereas in the *gsp1-2* gene product the changes are I61→F, W66→R, and V103→A. Separation of the L110→S and A135→V mutations from H50→L in *gsp1-1* resulted in loss of the conditional phenotype, suggesting that the temperature sensitivity of the mutant strains resulted from the cumulative effect of the three point mutations (data not shown). Because three point mutations were required to generate the conditional phenotype, it is not surprising that conditional alleles were only isolated under highly mutagenic conditions and that only single isolates of each were obtained.

Overexpression of Ntf2p partially rescues the temperature-sensitive phenotypes of *gsp1-1* and *gsp1-2*. What is the basis for

the temperature-sensitive growth of mutant *GSP1* alleles? One possibility is that mutant proteins are unstable at the nonpermissive temperature. In the case of *gsp1-1* and *gsp1-2*, the protein levels of mutant Gsp1p do not appear to be grossly affected at the nonpermissive temperature (data not shown). Another possibility which may cause conditional growth is a decreased interaction between the mutant gene product and one or more interacting proteins. Overexpression of the interacting protein may be able to shift the equilibrium from the noninteracting state to the interacting state. In order to identify what interaction(s) may be weakened with the mutant *GSP1* alleles, we overexpressed several known nuclear transport factors. We tested *YRB1*, *YRB2*, *RNA1*, *PRP20*, *KAP95*/*RSL1*, *KAP104*, *KAP121*/*PSE1*, *KAP123*, and *NTF2*. Of these, only *NTF2* could suppress the temperature-sensitive phenotype of *gsp1-1* and *gsp1-2* (Fig. 5A). Overexpressing *NTF2*, however, cannot rescue a *GSP1* knockout strain, indicating that the suppression by *NTF2* is not via a bypass mechanism (78a).

We tested whether overexpressing *NTF2* could also suppress the nuclear transport defects in mutant *GSP1* strains. Cells containing a high-copy *NTF2* plasmid or a control plasmid were tested by using in situ hybridization and indirect immunofluorescence microscopy. When mutant cells contained a high-copy *NTF2* plasmid, rescue of some of the temperature-sensitive phenotypes was observed. In *gsp1-1* cells, less than 25% of the cells exhibit a poly(A)$^+$ RNA export defect at the restrictive temperature (Fig. 5b, compare panels A and D). However, overexpression of *NTF2* could not rescue the Npl3p mislocalization defect (Fig. 5c, panel D). In constrast, the import defect observed in *gsp1-2* cells is rescued: in most of the cells, Npl3p is localized to the nucleus (Fig. 5c, panel J). Similar results were observed with the H2B-β-gal reporter construct (data not shown). Together, these data suggest that overexpression of Ntf2p can overcome many of the molecular defects in *gsp1-1* and *gsp1-2*.

Mutant Gsp1p has altered affinity for recombinant Ntf2p. Overexpression studies suggested that mutant Gsp1p proteins have a weakened interaction with Ntf2p. To test this hypothesis, we assayed the Ntf2p-Gsp1p interaction using bacterially expressed yeast Ntf2p bound to Sepharose beads. Previous work has shown that rat Ntf2p-Sepharose is able to bind both bacterially expressed Ran and Ran from rat liver homogenates (17, 21). Although immunoblots demonstrated that Ntf2p-Sepharose was able to bind wild-type Gsp1p from yeast lysates, neither Gsp1-1p nor Gsp1-2p bound significantly under these conditions (Fig. 6, lanes 2 and 3), suggesting that the interaction between Ntf2p and the mutated proteins is weaker than that between Ntf2p and wild-type Gsp1p. The interaction between Ntf2p-Sepharose and the nucleoporin Nsp1p is unaffected in wild-type and mutant lysates (Fig. 6, lanes 2 and 3).

The inability of mutated Gsp1p to bind to Ntf2p-Sepharose could be attributed to differences in affinity for guanine nucleotide. In fact, both Gsp1-1p and Gsp1-2p fail to bind either GTP or GDP in vitro (78a). A caveat to this interpretation is that the mutated proteins may be more susceptible to denaturation during the purification and therefore fail to bind guanine nucleotide.

Mutant Ntf2p proteins have altered interactions. Previously, we isolated conditional alleles of *NTF2* which exhibit nuclear import defects at the nonpermissive temperature (23). To characterize the molecular basis for their defects, we mapped the mutations to the recently solved crystal structure of rat Ntf2p (17). We used the rat structure because the structure of yeast Ntf2p has not yet been solved. However, when yeast Ntf2p was modeled on rat Ntf2p, the two structures were found to be very similar (23a). In addition, human Ntf2p (which is identical to rat Ntf2p) can functionally substitute for yeast Ntf2p, indicating that the two molecules are sufficiently structurally conserved to be able to interact with other components of the yeast nuclear transport machinery (23).

Point mutations in Ntf2p map to different sides of the molecule (Fig. 7). The mutation in Ntf2-1p (M83→T) is located in the interface between molecules in the dimer. The mutation in Ntf2-2p (D91→G) occurs in a loop which has been hypothesized to interact with Ran (17). Thus, *ntf2-2* strains may be temperature sensitive due to a weakened interaction with Ran. To test this hypothesis, we used a two-hybrid assay (33) to measure the interactions between wild-type and mutant Ntf2p and Gsp1p. To validate this approach, we tested whether or not two-hybrid constructs for *NTF2* and *GSP1* interact as they do in biochemical assays. Various studies have shown that Ntf2p forms a dimer (14, 49, 59). In a two-hybrid assay, Ntf2p interacts with itself indicating that Ntf2p also dimerizes in yeast (Table 1). Not only do yeast and human Ntf2p interact with themselves, they also interact with each other. Also, in agreement with biochemical data, human Ran can interact with human Ntf2p (Table 1) (17, 49, 55, 59). It is also interesting to note that both Gsp1p and Ran can interact with either yeast or human Ntf2p, indicating that their interaction is conserved between species (Table 1). The interaction between yeast and human Ntf2p and Gsp1p and Ran is specific since Ntf2p does not interact with another small GTPase, Ras, nor does Gsp1p interact with itself or Ran (Table 1). Thus, Ntf2p and Gsp1p interact appropriately in the two-hybrid system.

Having validated this approach, we tested the interaction between the mutant Ntf2p proteins and Gsp1p in the two-hybrid system. In the case of the Ntf2p mutants, differences in interaction are observed. Both proteins are expressed at approximately equal levels and can interact with Nsp1p (Table 2). As predicted, the interaction between Ntf2-2p and Gsp1p is lost while the interaction between Ntf2-1p and Gsp1p is unaffected (Table 2).

Overexpression of *ntf2-1* but not *ntf2-2* can suppress conditional *GSP1* alleles. It is possible that the overexpression of *NTF2* rescues the temperature-sensitive phenotype indirectly through an interaction with other components of the nuclear transport machinery and not with Gsp1p directly. In order to test if the direct interaction between Ntf2p and mutant Gsp1p proteins was indeed the cause of the rescue, we overexpressed the mutant *NTF2* alleles in wild-type and mutant *GSP1* strains. The predicted outcome is that only overexpression of *ntf2-1*, a mutant which can still interact with Gsp1p, would be able to rescue the temperature sensitivity of *GSP1* mutants. The results were as predicted: only overexpression of *ntf2-1* could rescue mutant *GSP1* alleles (Fig. 8). Overexpression of *ntf2-1* could rescue the transport defects in mutant *GSP1* strains to the same extent as overexpression of wild-type *NTF2* (data not shown).

DISCUSSION

Within the past few years, the mechanism by which macromolecules traverse the nuclear envelope has begun to be understood. In vitro assays which reconstitute protein import into the nucleus have been instrumental in identifying factors that are required for this process. One such factor is the Ras-like GTPase Ran (46, 50). To understand how Ran regulates nuclear transport in vivo, we have generated two conditional alleles of *GSP1*, a gene which encodes a Ran homolog, in the yeast *S. cerevisiae*. Both *gsp1-1* and *gsp1-2* exhibit nuclear transport defects. Overexpression of Ntf2p can rescue the temper-

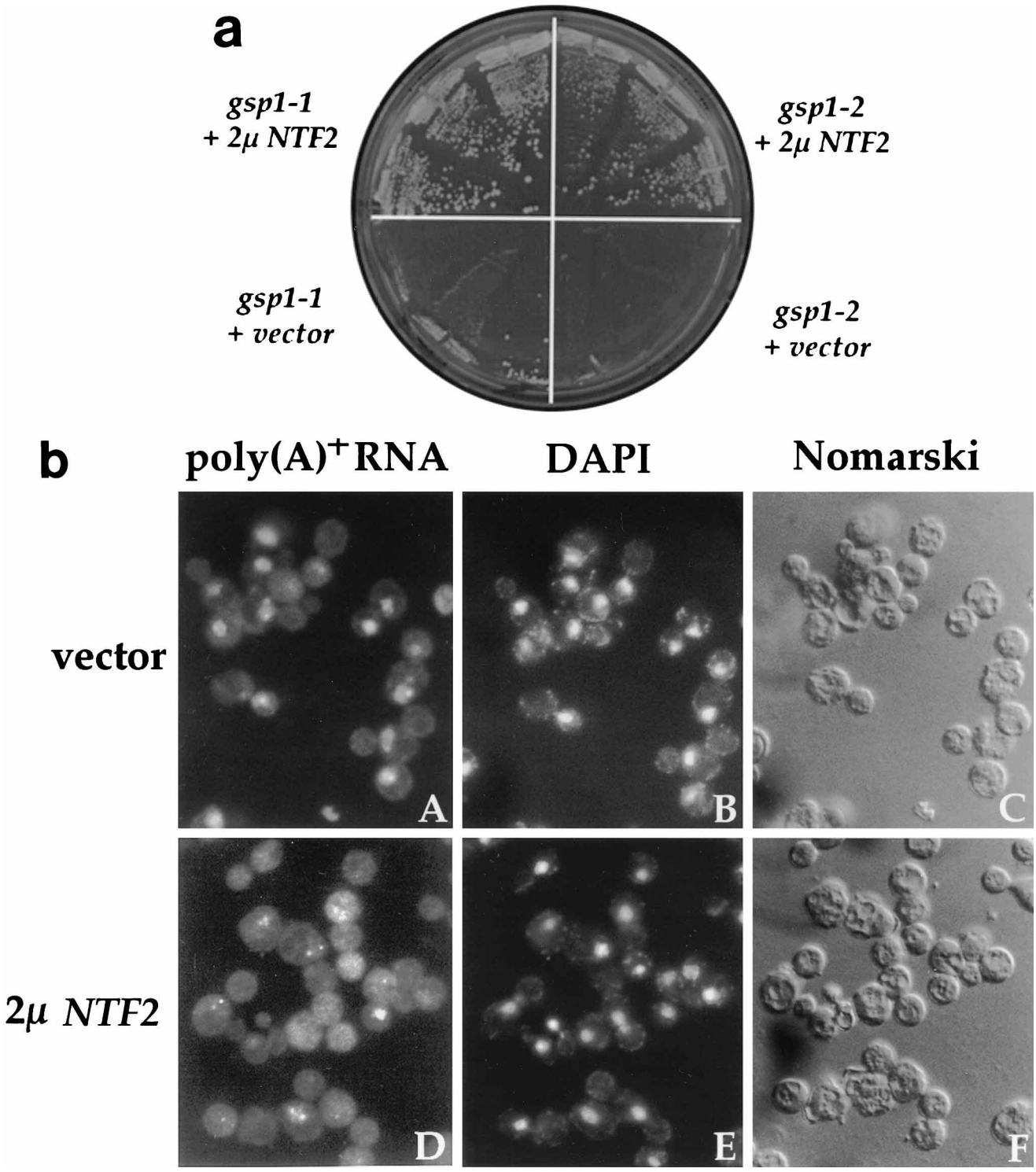

FIG. 5. Overexpression of *NTF2* partially rescues temperature-sensitive defect of *gsp1-1* and *gsp1-2*. (a) *NTF2* overexpression rescues temperature-sensitive growth defect. *GSP1*, *gsp1-1*, and *gsp1-2* strains were transformed with pPS992 (2μm *LEU2-NTF2*) (23) or YEp351 (2μm-*LEU* control vector) (35), restreaked to SC plates lacking leucine, and incubated at 25 or 36°C. (b) High-copy *NTF2* rescues the poly(A)$^+$ RNA export defect in *gsp1-1* cells. *gsp1-1* cells containing a vector control (YEp351) or pPS992 (2μm *LEU2-NTF2*) were grown to approximately 10^7 cells/ml in SC medium lacking leucine and shifted to 36°C for 3 h or left at room temperature. Cells were prepared for in situ hybridization. poly(A)$^+$ RNA in *gsp1-1* strains grown at 36°C containing a control vector (panel A) or 2μm *NTF2* (panel D) are shown along with the accompanying DAPI images (panels B and E) and Nomarski images (panels C and F). (c) Overexpression of *NTF2* can rescue the import defect in *gsp1-2* but not *gsp1-1* cells. *gsp1-1* (panels A to F) or *gsp1-2* (panels G to L) cells containing a vector control (panels A to C and G to I) or 2μm *NTF2* plasmid (panels D to F and J to L) were grown to approximately 10^7 cells/ml as described above and then shifted to 36°C for 1 h or left at room temperature. Cells were processed for indirect immunofluorescence microscopy with anti-Npl3p. Panels B, E, H, and K are the accompanying DAPI images, and panels C, F, I, and L are the accompanying Nomarski images.

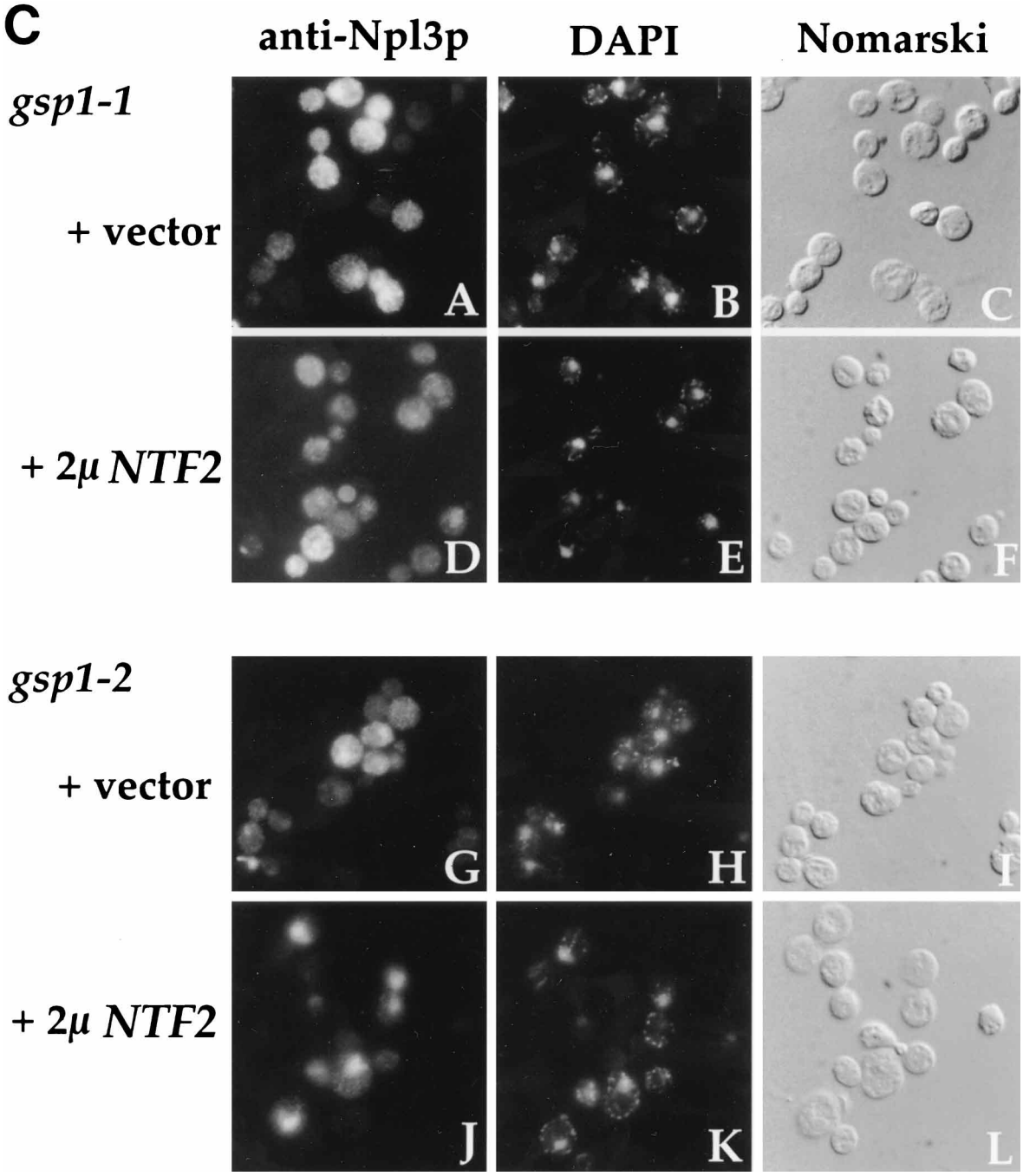

FIG. 5—*Continued.*

ature-sensitive phenotype, but overexpression of a mutant allele of *NTF2* with a decreased affinity for Gsp1p cannot rescue the temperature-sensitive phenotype of mutant *GSP1* alleles, emphasizing the importance of this interaction for the proper function of the nuclear protein import machinery.

Implications for other Ras-like GTPases. The isolation of temperature-sensitive alleles of *GSP1* has been a difficult process, and prior to this study, no conditional alleles existed. Temperature-sensitive alleles of *GSP1* were obtained only under highly mutagenic conditions and in a genetic background where *GSP2* was absent. In our genetic screen, only two single isolates were obtained. Each mutant contained three mutations in the coding region, all of which appear to be important for temperature sensitivity. Thus, generating conditional alleles of *GSP1* is a rare event.

Ran is a member of the Ras superfamily of small GTPases. The Ras-like GTPases all have a common G domain constructed from a central six-stranded β sheet surrounded by five α helices and connected by a common pattern of loops (reviewed in reference 71). This G domain contains the binding site for guanine nucleotides and undergoes a significant conformational change between GTP- and GDP-bound forms. A

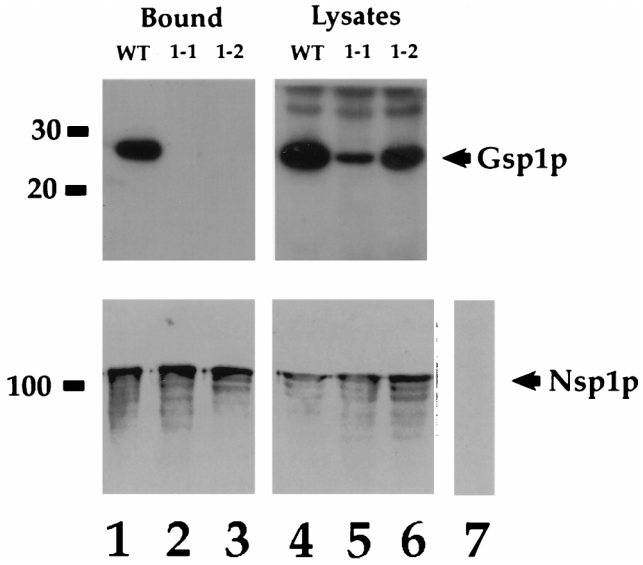

FIG. 6. Mutant Gsp1p proteins have altered affinity for Ntf2p. Lysates were prepared from *GSP1*, *gsp1-1*, or *gsp1-2* cells. Proteins from these lysates which bound recombinant yeast Ntf2p immobilized on CNBr-Sepharose beads were analyzed by immunoblotting with anti-Gsp1p antisera (gift of P. Belhumeur and M. Clark, University of Toronto) (lanes 1 to 3). Total lysates were also analyzed by immunoblotting (lanes 4 to 6). Lanes 1 and 4, wild-type lysates; lanes 2 and 5, *gsp1-1* lysates; lanes 3 and 6, *gsp1-2* lysates. Lysates were also immunoblotted with an antisera against the repeat region of Nsp1p. In wild-type and mutant *GSP1* strains, Nsp1p can interact with Ntf2p-Sepharose. The Nsp1p which binds to Ntf2p-Sepharose is specific since no Nsp1p is found bound to bovine serum albumin-Sepharose (lane 7).

range of other proteins interact with these GTPases, often involving the loop regions. Moreover, the binding of these proteins is often modulated by the nucleotide state of the GTPase. Although conditional mutants of some members of the Ras family of GTPases have been described, these mutations are not located in the regions of the G domain that are strongly conserved (68). In contrast, the mutations in *gsp1-1* and *gsp1-2* described in the present study are located in the β sheets that form the backbone of the G domain. Consequently, these *GSP1* mutants may form the basis for constructing conditional alleles of Ras and other members of the Ras superfamily.

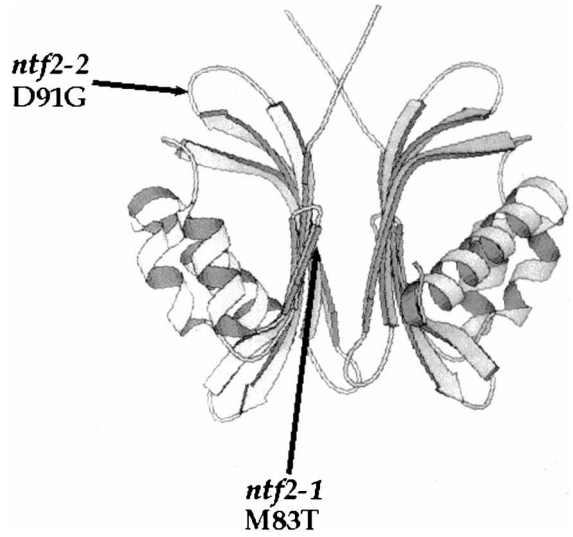

FIG. 7. Location of *ntf2-1* and *ntf2-2* mutations mapped on rat Ntf2p. Schematic illustration of the rat Ntf2p dimer as a ribbon diagram by using the program MOLSCRIPT (41). The yeast *ntf2-1* mutation, Met83 (M83T) lies in the β sheet that forms the interface between the two chains in the dimer, whereas the *ntf2-2* mutation, Asp91 (D91G) lies in the surface loop that appears to be involved in Ran binding (17).

TABLE 1. Interaction between Ntf2p and Ran across species is conserved[a]

Product expressed by pJG4-5 construct	pEG202 construct expression of:				
	yNtf2p	hNtf2p	Gsp1p	Ran	Ras
yNtf2p	+	+	+	+	−
hNtf2p	+	+	+	+	−
Gsp1p	+	+	−	−	ND
Ran	+	+	−	ND	ND

[a] pEG202 constructs (columns) were transformed into strain EGY48 and pJG4-5 constructs (rows) were transformed into strain EGY42. Strains were then mated, and diploids were selected. An interaction is scored as positive by two criteria: Leu^+ prototrophy and β-galactosidase activity (see Materials and Methods). +, OD_{420} 0.2 to 0.4; −, $OD_{420} < 0.05$ (β-galactosidase assay); ND, not done.

Mutant *GSP1* alleles exhibit nuclear transport defects. There is emerging evidence that different classes of nuclear proteins enter the nucleus via different mechanisms. Nuclear proteins containing classical NLSs (e.g., SV40 and H2B) enter the nucleus via the importin-α–importin-β pathway (19, 32). Npl3p, a yeast hnRNP, is a protein which rapidly shuttles in and out of the nucleus but does not contain any sequence motif resembling a classical NLS. Also, there is evidence suggesting that Npl3p enters the nucleus via a mechanism distinct from the classical NLS receptor. Conditional alleles of *SRP1* and *RSL1*, the yeast homologs of importin-α and importin-β, respectively, do not mislocalize Npl3p at the nonpermissive temperature although they do mislocalize proteins containing classical NLSs (40, 44). Additional evidence for a distinct import pathway comes from work with the mammalian shuttling protein hnRNP A1. This protein does not have a classical NLS. Instead, it has a bifunctional import-export signal known as an M9 sequence (47, 76). A different receptor, termed transportin, mediates its import (64). Transportin is an approximately 100-kDa protein that is distantly related to importin-β and is involved in the import of hnRNP A1. Like Npl3p import, this process does not appear to require importin-α (64).

To determine whether or not these two classes of protein are dependent on Ran for import into the nucleus, we localized both types of protein in mutant *GSP1* strains. In these strains, we demonstrate the rapid onset of a protein import defect upon shift to the nonpermissive temperature. In the case of the

TABLE 2. Interactions between mutant yeast Ntf2p and Gsp1p[a]

Product expressed by pJG4-5 construct	pEG202 construct expression of:			
	Ntf2p	Ntf2-1p	Ntf2-2p	Gsp1p
Ntf2p	+	−	+	+
Ntf2-1p	−	−	−	+
Ntf2-2p	+	−	−	−
Gsp1p	+	+	−	−
Nsp1p	+	+	+	+

[a] pEG202 constructs (columns) were transformed into strain EGY48 and pJG4-5 constructs (rows) were transformed into strain EGY42. Strains were then mated, and diploids were selected. An interaction is scored as positive by two criteria: Leu^+ prototrophy and β-galactosidase activity (see Materials and Methods). +, OD_{420} of 0.2 to 0.4; −, $OD_{420} < 0.05$.

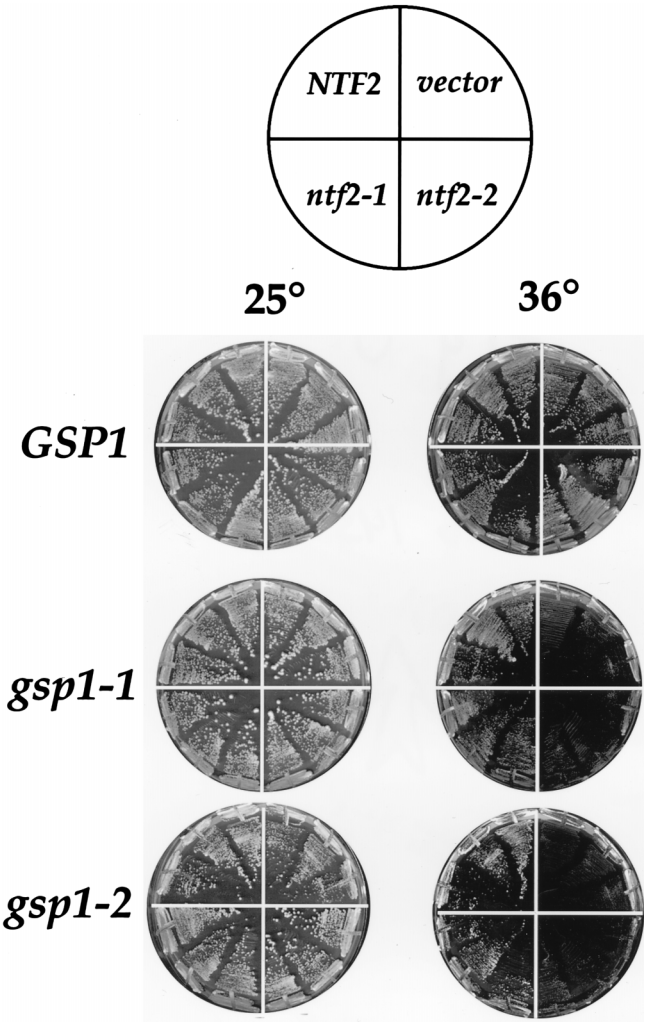

FIG. 8. High-copy *ntf2-1* but not *ntf2-2* can suppress the temperature-sensitive phenotype of mutant *GSP1* alleles. *GSP1*, *gsp1-1*, and *gsp1-2* strains were transformed with 2μm-*LEU2* versions of *NTF2* (pPS1023), *ntf2-1* (pPS1024), or *ntf2-2* (pPS1025) or a control plasmid (pRS425). Three colonies from each transformation were restreaked to SC plates lacking leucine and incubated at 25 or 36°C.

classical NLS, we examined the localization of an artificial reporter construct consisting of the NLS from the yeast histone 2B (H2B) fused to β-galactosidase (53). In the case of the nonclassical NLS, we used Npl3p (15, 26). In both cases, protein import into the nucleus was inhibited at the nonpermissive temperature in mutant *GSP1* alleles, indicating that although the mechanisms by which these proteins enter are different, both pathways involve Ran.

In addition to importin-β, three additional importin-β homologs have been identified in the *S. cerevisiae* genome. *KAP104* appears to be a yeast homolog of the mammalian transportin (4, 64). The other two importin-β homologs are *PSE1* and *KAP123. PSE1* was previously identified as a multicopy enhancer of protein secretion (20), whereas *KAP123* is a previously uncharacterized open reading frame. The roles of Pse1p and Kap123p in nuclear transport have not been clearly established. Once these roles have been determined, and if they indeed function at all in nuclear transport, it will be important to see if Ran is also involved in Pse1p- and Kap123p-mediated transport.

The observations above have important implications for how Ran functions in the cell. Ran has been implicated in a variety of processes including protein import, RNA export, mitotic regulation, and cell cycle progression (reviewed in references 67 and 70). Ran may affect all these processes directly or it may only affect a subset, the others being downstream of the initial defect. Given the rapid onset of the nuclear transport defects and the lack of any other noticeable phenotype, our observations with these particular alleles of *GSP1* suggest the latter model. However, we cannot rule out the possibility of isolating mutant alleles of *GSP1* which affect processes other than nuclear transport.

A direct interaction between Gsp1p and Ntf2p is required for nuclear import in vivo. We used biochemical and genetic approaches to address the way in which the temperature sensitivity of *gsp1-1* and *gsp1-2* was manifested. The ability of the mutant alleles to grow at the permissive temperature indicates that, under these conditions, the function of Gsp1p and especially its interactions with other components of the nuclear transport machinery is not grossly impaired.

Genetic studies suggested that the strength of the interaction between mutant Gsp1p and Ntf2p is reduced. We used biochemical as well as more directed genetic tests to further test this hypothesis at the molecular level. Biochemical experiments demonstrated that there is indeed a weakened interaction between Ntf2p and mutant Gsp1p proteins. This loss of binding appears to be independent of temperature. It is not unusual for the gene products of temperature-sensitive alleles to be nonfunctional in vitro even when prepared at the permissive temperature (22, 45, 48). Thus these in vitro observations most likely reflect changes in the interactions involving the mutant *GSP1* gene products in vivo at the nonpermissive temperature.

Although overexpression of *NTF2* can rescue the temperature-sensitive growth phenotype of both *gsp1-1* and *gsp1-2*, it may seem paradoxical that the protein import defect in *gsp1-1* is not suppressed at the nonpermissive temperature. One possible interpretation of this result is that the amount of nuclear import is sufficient to support growth albeit at a level too low to detect by immunofluorescence assays. Two observations support this explanation. First, nuclear import is already slightly impaired at the permissive temperature in *gsp1-1* cells, suggesting that a completely functional transport machinery is not required for viability. Slight nuclear transport defects have also been observed in other nuclear transport mutants at the permissive temperature (13). Second, overexpression of Ntf2p can rescue the poly(A)$^+$ RNA export defect, suggesting that there is partial rescue of protein import. Npl3p has been shown to be an important mediator of poly(A)$^+$ RNA export (43). Hence the transport of Npl3p in and out of the nucleus must be at least partially rescued in order to restore poly(A)$^+$ RNA export.

A model for Ntf2p function. Recently there has been controversy over the role of Ntf2p in nuclear import. In some studies using in vitro nuclear import assays, it is not necessary to add Ntf2p (18, 28, 30). However, we have previously shown that Ntf2p plays an essential role in nuclear import in vivo (23). How can we reconcile the difference between these in vitro and in vivo studies? A possible explanation for this discrepancy is that in some in vitro assay systems, residual Ntf2p remains in the permeabilized cells. Another possibility is that Ran has been added in excess of what is required for import, thus bypassing the need for Ntf2p. In fact, this situation can be mimicked in vivo as overexpression of *GSP1* can rescue a strain where *NTF2* is deleted (60).

In the present study, we demonstrate that the interaction

between Ntf2p and Gsp1p is required for nuclear import in vivo. The most compelling evidence for this interaction is the observation that overexpression of Ntf2-2p, a mutated protein with decreased interaction for Gsp1p, failed to rescue the temperature sensitivity of mutant *GSP1* strains. The most plausible mechanism to account for this observation would be that, at the nonpermissive temperature, the interaction between Ntf2p and the mutated Gsp1p proteins is reduced to such a low level that quantities of the Ntf2p-Gsp1p complex sufficient to enable adequate nuclear transport in the cells are not produced. Overexpression of Ntf2p would, through Le Châtelier's principle, increase the amount of complex formed and so enable adequate nuclear transport to take place.

How does Ntf2p function in concert with Gsp1p? Ntf2p has been shown to interact with the nucleoporin, p62 and has been localized to the nuclear pore (17, 21, 58, 59, 61). Ntf2p interacts with Ran-GDP as well as importin-β (59, 62). Thus Ntf2p may serve to direct the various nuclear transport factors to the nuclear pore and thus promote translocation through the pore.

This working hypothesis provides a framework for future experiments. These conditional alleles of *GSP1* will provide a valuable tool for an integrated approach of biochemistry, genetics, and cell biology to further dissect the nuclear transport machinery.

ACKNOWLEDGMENTS

We thank Claudio Saavedra for help with the in situ hybridization assay, Michelle Wien and Timothy Bullock for help with the structural analysis, and Gabriel Schlenstedt, W. David Clarkson, Paul Ferrigno and members of Pamela Silver's lab for helpful discussion and critical reading of this manuscript.

This work was supported by National Institutes of Health (NIH) grants to P. A. Silver. D. H. Wong was supported by a postdoctoral fellowship from the Sandoz Drug Discovery Program, and A. H. Corbett was supported by a Burroughs Wellcome Biomedical Career Development Award.

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