Saccharomyces cerevisiae Putative G Protein, Gtr1p, Which Forms Complexes With Itself and a Novel Protein Designated as Gtr2p, Negatively Regulates the Ran/Gsp1p G Protein Cycle Through Gtr2p

Nobutaka Nakashima, Eishi Noguchi and Takeharu Nishimoto

Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582, Japan

Manuscript received September 24, 1998
Accepted for publication March 18, 1999

ABSTRACT

Prp20p and Rna1p are GDP/GTP exchanging and GTPase-activating factors of Gsp1p, respectively, and their mutations, *prp20-1* and *rna1-1*, can both be suppressed by *Saccharomyces cerevisiae gtr1-11*. We found that *gtr1-11* caused a single amino acid substitution in Gtr1p, forming S20L, which is a putative GDP-bound mutant protein, while Gtr1p has been reported to bind to GTP alone. Consistently, *gtr1-S20N*, another putative GDP-bound mutant, suppressed both *prp20-1* and *rna1-1*. On the other hand, *gtr1-Q65L*, a putative GTP-bound mutant, was inhibitory to *prp20-1* and *rna1-1*. Thus, the role that Gtr1p plays *in vivo* appears to depend upon the nucleotide bound to it. Our data suggested that the GTP-bound Gtr1p, but not the GDP-bound Gtr1p, interacts with itself through its C-terminal tail. *S. cerevisiae* possesses a novel gene, *GTR2*, which is homologous to *GTR1*. Gtr2p interacts with itself in the presence of Gtr1p. The disruption of *GTR2* suppressed *prp20-1* and abolished the inhibitory effect of *gtr1-Q65L* on *prp20-1*. This finding, taken together with the fact that Gtr1p-S20L is a putative, inactive GDP-bound mutant, implies that Gtr1p negatively regulates the Ran/Gsp1p GTPase cycle through Gtr2p.

SACCHAROMYCES cerevisiae Gsp1p is a homologue of mammalian Ran (Ras-like nuclear G protein, Bischoff and Ponstingl 1991; Belhumeur *et al.* 1993). The nucleotide exchange of Gsp1p is carried out by the *S. cerevisiae* RCC1 homologue Prp20p, and the intrinsic GTPase of Gsp1p is activated by Rna1p (reviewed by Sazer 1996; Seki *et al.* 1996). Ran was found to be essential for nuclear protein import (Moore and Blobel 1993). Indeed, all the temperature-sensitive mutants (Ts^-) of Gsp1p show a defect in nuclear localization signal (NLS)-dependent nuclear protein import (Wong *et al.* 1997; Oki *et al.* 1998). Thus, Ran plays an important role in the nucleus/cytosol exchange of macromolecules (Moore and Blobel 1994; Melchior and Gerace 1995; Avis and Clarke 1996; Görlich and Mattaj 1996; Nigg 1997; Görlich 1998).

On the basis of an analogy with the Ras family (Boguski and McCormick 1993), events downstream of Ran/Gsp1p should be carried out by proteins that bind specifically to GTP-Ran/Gsp1p. The proteins that have been so far reported to bind to GTP-Ran/Gsp1p are the RanBP1 family comprising Yrb1p, Yrb2p, RanBP1, RanBP2/NUP358, and RanBP3 (Coutavas *et al.* 1993; Bischoff *et al.* 1995; Dingwall *et al.* 1995; Melchior *et al.* 1995; Schlenstedt *et al.* 1995; Wu *et al.* 1995; Yokoyama *et al.* 1995; Chi *et al.* 1996; Noguchi *et al.* 1997; Mueller *et al.* 1998; Taura *et al.* 1998), the importin β family (Görlich *et al.* 1997; Ullman *et al.* 1997), Dis3p (Noguchi *et al.* 1996; Shiomi *et al.* 1998), and RanBPM (Nakamura *et al.* 1998). The RanBP1 and importin β families are both involved in the nucleus/cytosol exchange of macromolecules, but the others are not.

The phenotype caused by a defect in the nucleotide exchange of Ran is pleiotropic. Cultures of the tsBN2 cell line, hamster *rcc1* (Uchida *et al.* 1990), show G_1 arrest at 39.5°, the nonpermissive temperature, if shifted before the S phase (Nishitani *et al.* 1991). From the S phase onwards, however, tsBN2 cells prematurely enter mitosis, resulting in premature chromatin condensation at 39.5° (Nishitani *et al.* 1991). The *Schizosaccharomyces pombe rcc1* homologue, *pim1*, shows a defect in chromosome decondensation (Sazer and Nurse 1994). Furthermore, the *S. cerevisiae RCC1* homologue, *PRP20*, was identified as a mutant defective in the mating process (*srm1-1*, Clark and Sprague 1989), mRNA splicing (*prp20-1*, Aebi *et al.* 1990), and mRNA export (*mtr1-2*, Kadowaki *et al.* 1993). It is still obscure whether these phenotypes of the *rcc1*/*pim1*/*prp20* mutants were caused by a defect in the nucleus/cytosol exchange of macromolecules or in other unknown pathways. To clarify this issue, we previously isolated the cold-sensitive mutants *ded1-21* (Hayashi *et al.* 1996) and *gtr1-11* (Nakashima *et al.* 1996) as suppressors of *srm1-1* and *mtr1-2*, respectively. *ded1-21* also suppresses *mtr1-2* (Kadowaki *et al.* 1993), but not *prp20-1* (Aebi *et al.* 1990). In contrast, *gtr1-11* suppresses not only all the *S. cerevisiae prp20* al-

Corresponding author: Takeharu Nishimoto, Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail: tnishi@molbiol.med.kyushu-u.ac.jp

leles, but also *rna1-1*, which encodes a Ts$^-$ form of Rna1p (Hutchison *et al.* 1969; Hopper *et al.* 1978; Amberg *et al.* 1992; Forrester *et al.* 1992).

GTR1 is not essential for survival, but its loss leads to cold-sensitive growth (Bun-ya *et al.* 1992). Gtr1p, a protein of 36 kD, possesses three motifs conserved in the Ras family, but has been reported to bind to GTP alone (Bun-ya *et al.* 1992). For this reason, Gtr1p is considered to be a putative G protein. Recently, Schurmann *et al.* (1995) identified two human cDNA clones, encoding proteins designated as RagA and RagB, that are homologous to Gtr1p and that rescue the cold-sensitive growth of the *gtr1-11* strain (Hirose *et al.* 1998). Thus, Gtr1p is conserved through evolution. RagA has been identified independently as a protein that interacts with the adenovirus 14.7-kD E3 protein (E3-14.7K, Li *et al.* 1997), and it may be involved in cell death.

In this article, we found that *gtr1-11* possessed a single amino acid substitution, S20L, that corresponds to a GDP-bound mutant of G proteins. Consistent with this finding, both *prp20-1* and *rna1-1* were suppressed by another putative GDP-bound mutant of Gtr1p, *gtr1-S20N*, but not by the putative GTP-bound mutant *gtr1-Q65L*. Overexpression of Gtr1p-Q65L was rather inhibitory for colony formation of *prp20-1* and *rna1-1* cells. We found a novel protein homologous to Gtr1p, designated as Gtr2p, that formed complexes with Gtr1p. Interestingly, the disruption of *GTR2* suppressed *prp20-1* and abolished the inhibitory effect of *gtr1-Q65L* on *prp20-1*.

MATERIALS AND METHODS

Strains and media: All the *S. cerevisiae* strains and plasmids used in this study are described in Tables 1 and 2, respectively. They were constructed by standard genetic manipulations (Kaiser *et al.* 1994). Transformation of *S. cerevisiae* was carried out by a modified LiCl method using DMSO (Hill *et al.* 1991). The media used for *S. cerevisiae* and bacteria have been described previously (Nakashima *et al.* 1996).

Site-directed mutagenesis: The 3.4-kb *Cla*I/*Bam*HI fragment of pL3 (Nakashima *et al.* 1996) was inserted into the *Cla*I/*Bam*HI sites of the pBluescript II SK(+) (Ichihara and Kurosawa 1993). The resultant pL51 was mutagenized with the Kunkel's site-directed mutagenesis method by means of the site-directed mutagenesis system Mutan-K (Takara, Kyoto, Japan) using as a mutagenic oligonucleotide 5′ TGTGGTGGGCT CGACGTGTTTATG 3′ for Q65L mutation, 5′ ATGGGCCGGG TCGGCTCCGGTAAATCGTCA for S15V, 5′ ATGGGCCGGG GCGGCTCCGGTAAATCGTCA for S15G, 5′ GGCTCCGGTA AAAACTCAATGAGGT for S20N, or 5′ CGCCCTTGAAATT TGACTTTTCGAAAGAACAAC 3′ for deletion of the C-terminal region of Gtr1p.

Construction of *GTR1* and *GTR2* plasmids: The 5.2-kb *Xba*I/*Sal*I fragment was cut out from pL3 (Nakashima *et al.* 1996) and inserted into the *Xba*I/*Sal*I sites of pUC29, resulting in pL6. The 1.5-kb *GTR1* fragment carried on the resultant pL6 plasmid was amplified by PCR using 5′ CAATTTAGCCATGGC GTCAAATAATAGGAAG 3′ and M13 Reverse (Toyobo, Osaka, Japan) as the primers and KOD Polymerase (Toyobo) as a polymerase. Amplified DNA fragments were digested with *Nco*I and *Bam*HI enzymes and inserted into the *Nco*I/*Bam*HI sites of pUC29, resulting in pL19. The 1.5-kb fragment was cut out from pL19 with *Nco*I and *Sal*I enzymes and inserted into the *Sal*I/*Nco*I sites of pGEX-KG (Guan and Dixon 1991), resulting in pL20. To express *GST*-fused *GTR1* in *S. cerevisiae*, 1.5 kb of *GST*-fused *GTR1* was cut out from pL20 by *Sma*I and *Sal*I enzymes and inserted into the *Sma*I/*Sal*I sites of pEG-KG (Mitchell *et al.* 1993), resulting in pL38.

The genomic DNA of the NBW5 strain was amplified using primers A and D as shown in Figure 6A, resulting in a 2.2-kb DNA fragment that was digested with *Bam*HI/*Sal*I enzymes and inserted into the *Bam*HI/*Sal*I sites of YEplac195. The 1.7-kb fragment of *GTR2* carried on the resulting plasmid pL130 was amplified by PCR using 5′ TACACCATGGGTTTAGAGGC TACAGATTCCAAGGCAATGC 3′ and M13 Reverse as the primers and KOD Polymerase as a polymerase. The amplified DNA fragments were digested with *Nco*I and *Sal*I enzymes and inserted into the *Nco*I/*Sal*I sites of pEG-KG (Mitchell *et al.* 1993), resulting in pL153.

The 1.6-kb fragment of *HA*-fused *GTR1* was cut out from the plasmid pL46 (Nakashima *et al.* 1996) by *Xho*I and *Sma*I enzymes and inserted into the *Xho*I/*Sma*I sites of the plasmid YEplac112-XA (Funakoshi *et al.* 1997), resulting in pL80.

The 1.7-kb *GTR2* fragment carried on pL130 was amplified as described above and inserted into the *Nco*I/*Sal*I sites of pUC29. From the resulting pL136, the 1.8-kb *GTR2* fragment was digested with *Nco*I and *Pst*I enzymes and inserted into the *Nco*I/*Pst*I sites of plasmid TKS-HA1 (Nakashima *et al.* 1996). From the resulting pL152, the 1.8-kb *GTR2* fragment was cut out with *Xho*I and *Sma*I enzymes and inserted into the *Xho*I/*Sma*I sites of YEplac112-XA (Funakoshi *et al.* 1997), resulting in pL154.

The 1.5-kb *GTR1* fragments were digested from pL20 and its derivatives containing mutated *gtr1* by the *Bam*HI enzyme and then inserted into the *Bam*HI site of pET-28a (Novagen Inc.), resulting in pL115 (wild type), pL117 (Gtr1-12Δp), and pL118 (Gtr1-13p). The 1.3-kb fragment containing *His6-T7* carried on pET-28a was digested by *Nco*I and *Sma*I enzymes and inserted into the *Nco*I/*Stu*I sites of pUC29, resulting in pL96. The 0.1-kb *His6-T7* fragment was digested from pL96 by the *Sac*I enzyme and inserted into the *Sac*I site of pEG-KG, resulting in pEH7-SH. The 1.5- and 1.7-kb *Bam*HI fragments containing *GTR1* and *GTR2* were then inserted into the *Bam*HI site of pEH7-SH, resulting in pL101 and pL210, respectively.

Construction of *GAL4 TAD*- and *DBD*-fused *GTR1*: For the two-hybrid assay (Fields and Song 1989), the *GAL4*-promoter activation domain (*TAD*) and the DNA-binding domain (*DBD*) were fused to the wild-type and mutated *GTR1* clones as follows. The 1.5-kb fragments of the wild-type and mutated *GTR1* were cut out from pL19 or its derivatives containing the mutated *gtr1* with *Nco*I and *Bam*HI enzymes and then inserted into the *Nco*I/*Bam*HI sites of pACT2 (Durfee *et al.* 1993).

The 1.2-kb *Sac*I/*Sal*I fragment of pAS1 (Durfee *et al.* 1993) containing the *ADH* promoter, the *GAL4* DNA-binding domain, and the *HA* epitope was inserted into the *Sac*I/*Sal*I sites of pRS404. Into the *Nco*I/*Bam*HI sites of the resulting pAS404, 1.5-kb *Nco*I/*Bam*HI fragments containing the wild-type and mutated *GTR1* were then inserted.

Disruption of *GTR2*: The genomic DNA of NBW5 was amplified using either primers A and B or primers C and D (see Figure 6A) to obtain the fragments AB and CD, respectively. The fragment AB was digested with *Bam*HI/*Xho*I enzymes and inserted into the *Bam*HI/*Xho*I sites of pUC29, resulting in pL119. The fragment CD was then digested with *Xho*I/*Sal*I enzymes and inserted into the *Xho*I site of pL119, resulting in pL126. Finally, the *LEU2* gene of YEp13 (Broach *et al.* 1979) was cut out with *Xho*I/*Sal*I enzymes and inserted into the *Xho*I site of pL126, resulting in pL128. The 3.4-kb fragment containing *LEU2::gtr2-1*Δ was cut out with *Bam*HI/*Nco*I enzymes from pL128 and then introduced into the strain N43.

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source or reference
NBW5	*MAT*α *ade2 ura3-1,2 his3-532 leu2-3,112 trp1-289 can1*	Matsuzaki *et al.* (1990)
NBW5ΔGTR1	*MAT*α *gtr1-1*Δ *ade2 ura3-1,2 his3-532 leu2-3,112 trp1-289 can1*	Nakashima *et al.* (1996)
NBW5ΔGTR2	*MAT*α *gtr2-1*Δ *ade2 ura3-1,2 his3-532 leu2-3,112 trp1-289 can1*	This study
NBW5ΔGTR1/ΔGTR2	*MAT*α *gtr1-1*Δ*gtr2-1*Δ *ade2 ura3-1,2 his3-532 leu2-3,112 trp1-289 can1*	This study
prp20/2c	*MAT***a** *prp20-1 ade2-101 ura3-52 his3-*Δ*200 lys2-801*	Aebi *et al.* (1990)
N43	*MAT*α *ade2 ura3-1,2 his3-532 leu2-3,112 trp1-289 + can1* / *MAT***a** *ade2 ura3-52 his3-*Δ*200 leu2-*Δ*1 trp1-*Δ*63 lys2-801*	Noguchi *et al.* (1997)
N43ΔGTR2	*MAT*α *gtr2-1*Δ *ade2 ura3-1,2 his3-532 leu2-3,112 trp1-289 + can1* / *MAT***a** *+ ade2 ura3-52 his3-*Δ*200 leu2-*Δ*1 trp1-*Δ*63 lys2-801*	This study
N43ΔGTR1,2	*MAT*α *gtr1-1*Δ *gtr2-1*Δ *ade2 ura3-1,2 his3-532 leu2-3,112 trp1-289 + can1* / *MAT***a** *+ + ade2 ura3-52 his3-*Δ*200 leu2-*Δ*1 trp1-*Δ*63 lys2-801*	This study
Y190	*MAT*α *gal4 gal80 ade2 his3-532 leu2 trp1 URA3::GAL1-lacZ LYS2::GAL1-lacZ*	Harper *et al.* (1993)
Y190-GTR1	*MAT*α *gal4 gal80 ade2 his3-532 leu2 trp1 URA3::GAL1-lacZ LYS2::GAL1-lacZ TRP1::ADH1-GAL4DBD-GTR1*	This study
Y190-gtr1-11	*MAT*α *gal4 gal80 ade2 his3-532 leu2 trp1 URA3::GAL1-lacZ LYS2::GAL1-lacZ TRP1::ADH1-GAL4DBD-gtr1-11* (S20L)	This study
Y190-gtr1-13	*MAT*α *gal4 gal80 ade2 his3-532 leu2 trp1 URA3::GAL1-lacZ LYS2::GAL1-lacZ TRP1::ADH1-GAL4DBD-gtr1-13* (Q65L)	This study
Y190-gtr1-12Δ	*MAT*α *gal4 gal80 ade2 his3-532 leu2 trp1 URA3::GAL1-lacZ LYS2::GAL1-lacZ TRP1::ADH1-GAL4DBD-gtr1-12*Δ (C-del.)	This study
Y190-gtr1-16	*MAT*α *gal4 gal80 ade2 his3-532 leu2 trp1 URA3::GAL1-lacZ LYS2::GAL1-lacZ TRP1::ADH1-GAL4DBD-gtr1-16* (S20N)	This study
Y190-gtr1-15	*MAT*α *gal4 gal80 ade2 his3-532 leu2 trp1 URA3::GAL1-lacZ LYS2::GAL1-lacZ TRP1::ADH1-GAL4DBD-gtr1-15* (G15V)	This study
NN7-3B	*MAT*α *gtr1-11 ade2 ura3 leu2*	Hirose *et al.* (1998)
NN19-5B	*MAT***a** *rna1-1 ade2 ura3 leu2 trp1*	Noguchi *et al.* (1997)
HS203	*MAT***a** *prp20-1 ade2 ura3 leu2 trp1*	Segregant from NBW5 × prp20/2c
HS203 ΔGTR1	*MAT***a** *prp20-1 gtr1-1*Δ *ade2 ura3 his3 leu2 trp1*	This study
HS203 ΔGTR2	*MAT***a** *prp20-1 gtr2-1*Δ *ade2 ura3 his3 leu2 trp1*	This study
HS203 ΔGTR1,2	*MAT***a** *prp20-1 gtr1-1*Δ *gtr2-1*Δ *ade2 ura3 his3 leu2 trp1*	This study

Purification of Gtr1p: GST- or His6-fused wild-type and mutated Gtr1p were expressed in *Escherichia coli* and purified as described by Noguchi *et al.* (1997) and Nuoffer *et al.* (1995), respectively.

Cosedimentation of HA-fused proteins with the GST-fused proteins: The plasmids carrying the *GST*- and *HA*-fused gene were cointroduced into the strain NBW5ΔGTR1. Transformant cultures were grown to OD_{660} = 1.0 in SR medium (0.67% yeast nitrogen base without amino acid, 2% raffinose) lacking uracil and tryptophan, and then 2% galactose was added. After incubation for 2 hr at 30°, the cells were spun down, washed once with distilled water, and then resuspended in S buffer [50 mm potassium phosphate, pH 6.5, 120 mm NaCl, 1 mm $MgCl_2$, 0.1% Triton X-100, 10% glycerol, 1 mm 2-mercaptoethanol, and 0.2 mm ρ-amidinophenyl-methanesulfonyl fluoride (ρ-APMSF)]. Cells were then frozen at −80° and disrupted by glass beads. After centrifugation at 83,500 × *g* for 30 min, the supernatant was mixed with glutathione Sepharose-4B beads that had been saturated with S buffer, and then rotated for 1 hr. The beads were spun down and washed five times with S buffer. Proteins bound to the beads were analyzed by SDS-PAGE and immunoblotting. All procedures were carried out at 4° except where otherwise indicated.

β-Galactosidase assay: Overnight cultures of transformants of Y190 (0.3 ml) expressing *GAL4-TAD*-fused clones and *GAL4-DBD*-fused clones were inoculated into 15 ml of synthetic medium containing 0.67% yeast nitrogen base without amino acid, 2% ethanol, 3% glycerol, and the appropriate amino acids. Cultures were allowed to grow to OD_{660} = 0.9 and then spun down, washed once with distilled water, resuspended in 200 μl lysis buffer (0.1 m Tris-HCl, pH 8.0, 20% glycerol, 1 mm DTT, and 0.2 mm ρ-APMSF), and then frozen at −80°. Frozen cells were disrupted by beating with glass beads. After centrifugation at 83,500 × *g* for 30 min, the supernatant was used as the crude extract.

The enzyme assay was performed as reported (Kandels-Lewis and Seraphin 1993). The yeast crude extract (100 μl) or, as a control, the lysis buffer alone was mixed with 900 μl Z buffer (100 mm sodium phosphate buffer, pH 7.0, 10 mm KCl, 1 mm $MgSO_4$, and 50 mm 2-mercaptoethanol). After incubation for 1–2 min at 28°, 200 μl *o*-nitrophenyl-β-d-galactopyranoside (4 mg/ml) was added, and the mixture was incubated at 28°. The reaction was stopped by the addition of 4.8 ml of 1 m Na_2CO_3. The amount of *o*-nitrophenol produced was estimated by measuring A_{420}, and the protein content was estimated by the BCA Protein Assay kit (Pierce, Rockford, IL). One unit of β-galactosidase activity corresponds to the amount of β-galactosidase required to produce 1 nmol of *o*-nitrophenol in 1 min at 28°.

Immunoblotting: Proteins were loaded on 11% SDS-PAGE, transferred onto PVDF membrane filters, and then probed

TABLE 2

Plasmids used in this study

Plasmid	Markers	Comments	Source or reference
YEplac195	2μ *URA3*		Gietz and Sugino (1998)
pL63	2μ *URA3 GTR1*	YEplac195 containing *GTR1*	Nakashima *et al.* (1996)
pL64	2μ *URA3 gtr1-11*(S20L)	YEplac195 containing *gtr1-11* allele	Nakashima *et al.* (1996)
pL112	2μ *URA3 gtr1-13* (Q65L)	YEplac195 containing *gtr1-13* allele (as *Cla*I/*Bam*HI fragment; C_2/B_1)[a]	This study
pL111	2μ *URA3 gtr1-12*Δ (C-del)	YEplac195 containing *gtr1-12*Δ allele (as *Cla*I/*Bam*HI fragment; C_2/B_1)[a]	This study
pL167	2μ *URA3 gtr1-14*(S15G)	YEplac195 containing *gtr1-14* allele (as *Cla*I/*Bam*HI fragment; C_2/B_1)[a]	This study
pL168	2μ *URA3 gtr1-15*(S15V)	YEplac195 containing *gtr1-15* allele (as *Cla*I/*Bam*HI fragment; C_2/B_1)[a]	This study
pL169	2μ *URA3 gtr1-16*(S20N)	YEplac195 containing *gtr1-16* allele (as *Cla*I/*Bam*HI fragment; C_2/B_1)[a]	This study
pL130	2μ *URA3 GTR2*	YEplac195 containing *GTR2* (as *Sal*I/*Bam*HI fragment)	This study
pEG-KG	2μ *URA3 leu2-d GAL1-10:GST*		Mitchell *et al.* (1993)
pL38	2μ *URA3 leu2-d GAL1-10:GST-GTR1*	*GST*-fused *GTR1* for *S. cerevisiae* expression	This study
pL155	2μ *URA3 leu2-d GAL1-10:GST-GTR2*	*GST*-fused *GTR1* for *S. cerevisiae* expression	This study
YEplac 112-XA	2μ *TRP1GAL1-10:cyclinA1*	YEplac112 containing *Xenopus laevis cyclin A1*	Funakoshi *et al.* (1997)
pL80	2μ *TRP1GAL1-10:HA-GTR1*	*HA*-fused *GTR1* for *S. cerevisiae* expression	This study
pL154	2μ *TRP1GAL1-10:HA-GTR2*	*HA*-fused *GTR2* for *S. cerevisiae* expression	This study
pACT2	2μ *LEU2GAL4TAD*		Harper *et al.* (1993)
pL21	2μ *LEU2GAL4TAD-GTR1*	*GAL4TAD*-fused *GTR1* for two-hybrid assay	This study
pL71	2μ *LEU2GAL4TAD-gtr1-11*	*GAL4TAD*-fused *gtr1-11* for two-hybrid assay	This study
pL105	2μ *LEU2GAL4TAD-gtr1-13*	*GAL4TAD*-fused *gtr1-13* for two-hybrid assay	This study
pL86	2μ *LEU2GAL4TAD-gtr1-12*Δ	*GAL4TAD*-fused *gtr1-12*Δ for two-hybrid assay	This study
pL184	2μ *LEU2GAL4TAD-gtr1-15*	*GAL4TAD*-fused *gtr1-15* for two-hybrid assay	This study
pL105	2μ *LEU2GAL4TAD-gtr1-16*	*GAL4TAD*-fused *gtr1-16* for two-hybrid assay	This study
pL44	*TRP1GAL4DBD-GTR1*	*GAL4DBD*-fused *GTR1* for two-hybrid assay	This study
pL66	*TRP1GAL4DBD-gtr1-11*	*GAL4DBD*-fused *gtr1-11* for two-hybrid assay	This study
pL106	*TRP1GAL4DBD-gtr1-13*	*GAL4DBD*-fused *gtr1-13* for two-hybrid assay	This study
pL87	*TRP1GAL4DBD-gtr1-12*Δ	*GAL4DBD*-fused *gtr1-12*Δ for two-hybrid assay	This study
pL208	*TRP1GAL4DBD-gtr1-15*	*GAL4DBD*-fused *gtr1-15* for two-hybrid assay	This study
pL209	*TRP1GAL4DBD-gtr1-16*	*GAL4DBD*-fused *gtr1-16* for two-hybrid assay	This study
pMB130	*HIS3 gtr1-1*Δ *PHO84*	pUC119 containing *gtr1-1*Δ allele and *PHO84* (for gene disruption of *GTR1*)	Bun-ya *et al.* (1992)
pL128	*LEU2 gtr2-1*Δ	pUC29 containing *gtr2-1*Δ allele (for gene disruption of *GTR2*)	This study
pRS314	*CEN6 TRP1*		Sikorski and Hieter (1989)
pL139	*CEN6 TRP1 GTR1*	pRS314 containing *GTR1* (as *Kpn*I/*Bam*HI fragment; Kp/B_1)[a]	This study
pL148	*CEN6 TRP1gtr1-11*	pRS314 containing *gtr1-11* allele (as *Kpn*I/*Bam*HI fragment; Kp/B_1)[a]	This study
pL146	*CEN6 TRP1 gtr1-13*	pRS314 containing *gtr1-13* allele (as *Cla*I/*Bam*HI fragment; C_2/B_1)[a]	This study
pL176	*CEN6 TRP1 gtr1-14*	pRS314 containing *gtr1-14* allele (as *Cla*I/*Bam*HI fragment; C_2/B_1)[a]	This study
pL177	*CEN6 TRP1 gtr1-15*	pRS314 containing *gtr1-15* allele (as *Cla*I/*Bam*HI fragment; C_2/B_1)[a]	This study
pL178	*CEN6 TRP1 gtr1-16*	pRS314 containing *gtr1-16* allele (as *Cla*I/*Bam*HI fragment; C_2/B_1)[a]	This study
pL101	2μ *URA3 leu2-d GAL1-10:T7-GTR1*	*T7*-fused *GTR1* for immunofluorescence microscopy	This study
pL210	2μ *URA3 leu2-d GAL1-10:T7-GTR1*	*T7*-fused *GTR2* for immunofluorescence microscopy	This study
pET-28a			Novagen, Inc.
pL115		*His*6-fused *GTR1* for *E. coli* expression	This study
pL117		*His*6-fused *gtr1-12*Δ for *E. coli* expression	This study
pL118		*His*6-fused *gtr1-13*Δ for *E. coli* expression	This study

[a] Abbreviations are described in Nakashima *et al.* (1996)

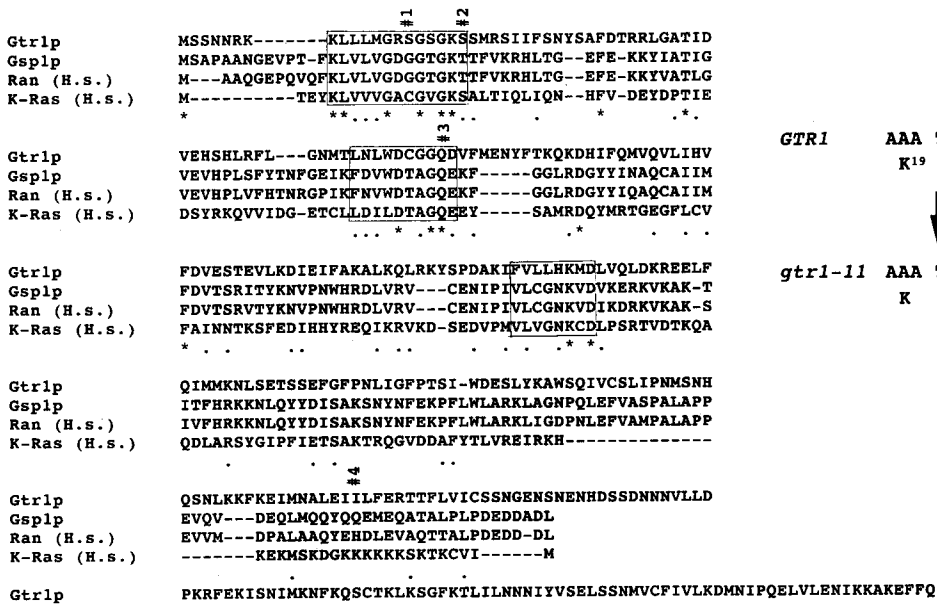


Figure 1.—Alignment of amino acid sequences of Gtr1p, Gsp1p, Ran, and K-Ras. Asterisks and periods indicate identical and chemically conserved amino acid residues, respectively. Also shown are the mutated sites of assumed GTP- (#1 and #3) and GDP- (#2) bound forms of Gtr1p. Inset: the mutation site of *gtr1-11*. Isoleucin of Gtr1p (#4) was changed to the stop codon to create the C-terminal deletion.

with the anti-HA mAb (Babco) or anti-T7 mAb (Novagen, Inc.) as described previously (Noguchi *et al.* 1997).

RESULTS

Mutation site of *gtr1-11*: To clarify how *gtr1-11*, a cold-sensitive mutation of *GTR1*, suppresses both *prp20* and *rna1*, we isolated the *GTR1* gene from the *gtr1-11* strain and determined its nucleotide sequence. In comparison with the wild-type *GTR1*, *gtr1-11* was found to have thymine instead of cytosine at the 20th codon of Gtr1p, and serine was thereby changed to leucine (S20L, Figure 1 insert). No other nucleotide substitution was found in the *gtr1-11* sequence. By comparison with other small G proteins, the 20th amino acid of Gtr1p, serine, was suggested to correspond to threonine, the 24th and 26th amino acid of Ran and Gsp1p, respectively (Figure 1). Hence, by analogy with the RanT24N mutant (Dasso *et al.* 1994), the S20L substitution of Gtr1p is likely to result in a dominant negative GDP-bound form of the protein, implying that the GDP-bound Gtr1p has some biological function in cells. To investigate the function of the Gtr1p, additional mutants that should be locked in the GTP-bound state (Gtr1p-Q65L, -S15V, and -S15G) or the GDP-bound state (Gtr1p-S20N) were constructed on the basis of mutants of Ras (Boguski and McCormick 1993). These mutated *gtr1* clones were inserted into either a single-copy vector, pRS314, or a multicopy vector, YEplac195 (Table 2).

When the *gtr1* mutants carried on a single-copy vector were expressed in the *gtr1-1*Δ strain (Figure 2A), two putative GTP-bound mutants, *gtr1-S15V* and *gtr1-S15G*, rescued the cold sensitivity of the *gtr1-1*Δ strain with an efficiency similar to that of wild-type *GTR1*, but another putative GTP-bound mutant, *gtr1-Q65L*, rescued it only weakly. On the other hand, both putative GDP-bound mutants, *gtr1-S20N* and *gtr1-S20L*, did not rescue the cold sensitivity of the *gtr1-1*Δ strain. Thus, both *gtr1-S15V* and *gtr1-S15G* behaved like wild types, whereas the other mutants did not. The mutated and wild-type *GTR1* clones carried on a multicopy vector were then introduced into the haploid strains prp20/2c (*prp20-1*), NN19-5B (*rna1-1*), and, as a control, NBW5 (wild type). Ura$^+$ transformants were selected and plated on synthetic medium lacking uracil at the three temperatures indicated (Figure 2B)—the permissive, semipermissive, and nonpermissive temperatures for each mutated strain.

The two putative GDP-bound mutants, *gtr1-S20L* and *gtr1-S20N*, both rescued the temperature sensitivity of each of the *prp20-1* and *rna1-1* strains, whereas a putative GTP-bound mutant, *gtr1-Q65L*, did not (Figure 2B). Interestingly, *gtr1-Q65L* inhibited the colony formation of both *prp20-1* and *rna1-1* cells at 30° and 28°, the semipermissive temperatures for each mutant. The other putative GTP-bound mutants, *gtr1-S15V* and *gtr1-S15G*, did not show any inhibitory effect on *prp20-1* and *rna1-1* cells. Thus, both *gtr1-S15V* and *gtr1-S15G* behaved like the wild types, consistent with the finding that these mutants effectively complemented *gtr1-1*Δ (Figure 2A).

Gtr1p binds not only to GTP, but also to GDP: *E. coli*-produced GST-fused Gtr1p was purified on glutathione Sepharose-4B beads. The purified GST-fused Gtr1p was mixed with either ^{3}H-labeled GTP or GDP. As a control, the nucleotide-binding experiments were also conducted in the presence of EDTA, which is reported to release the nucleotides from Ran (Bischoff *et al.* 1995; Richards *et al.* 1995). After incubation for 30 min at 30°, the glutathione Sepharose-4B beads were spun down, and the radioactivity that was coprecipitated with the beads was quantified using a liquid scintillation counter. *E. coli*-produced Gtr1p bound efficiently to [^{3}H]GTP, but it did not bind as well to [^{3}H]GDP when

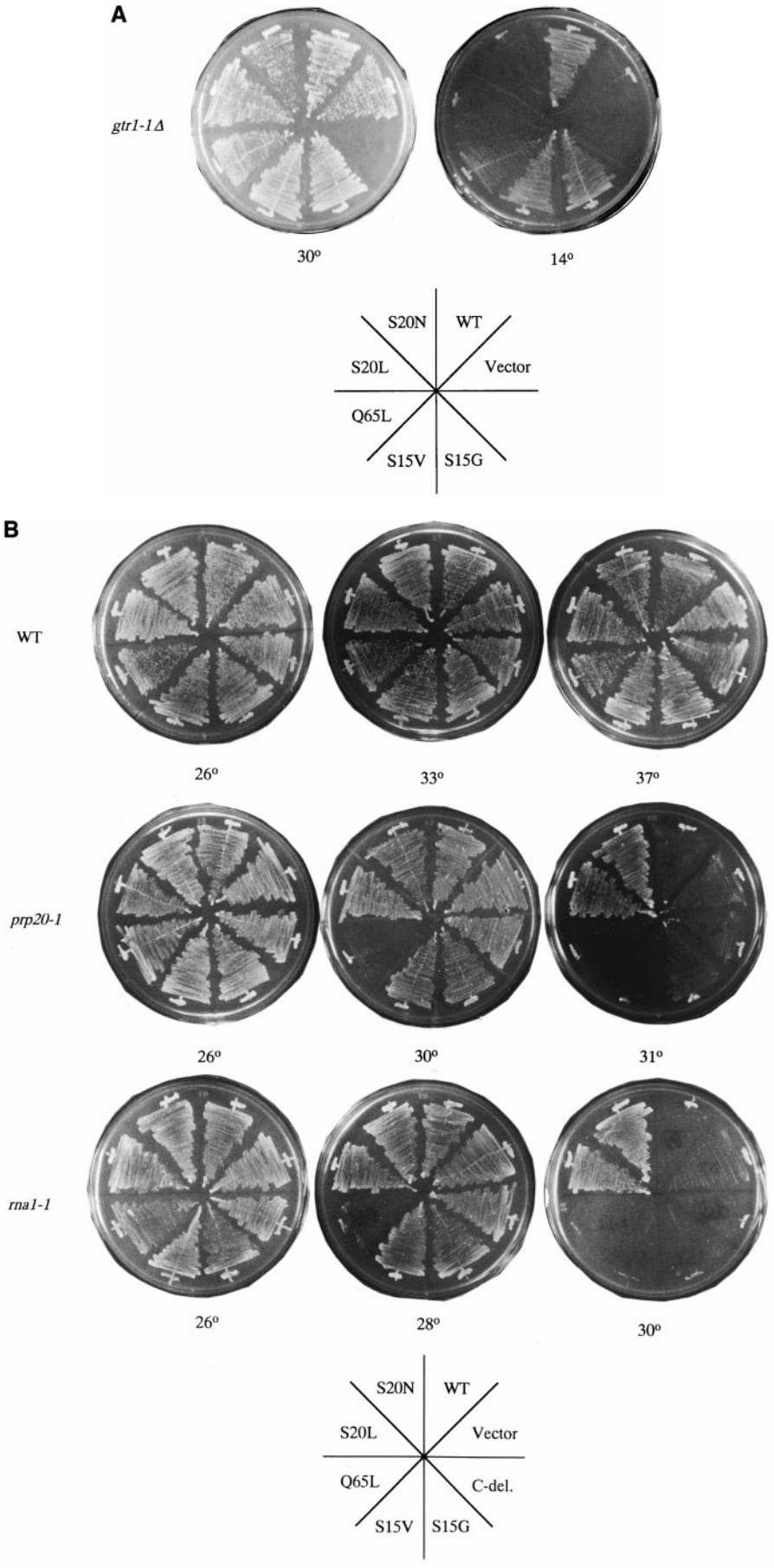


Figure 2.—Biological effect of the putative GTP- and GDP-bound forms of Gtr1p. (A) Single-copy plasmids carrying the indicated mutated and wild-type *GTR1* genes and, as a control, the vector pRS314 alone were introduced into strain *gtr1-1*Δ (NBW5ΔGTR1). The resulting Trp^+ transformants were plated on synthetic medium lacking tryptophan and were incubated at 14° or 30° as indicated. (B) YEplac195 plasmids carrying the indicated mutated and wild-type *GTR1* genes and, as a control, the vector alone were introduced into the indicated strains, wild-type (NBW5), *prp20-1* (prp20/2c) (Aebi *et al.* 1990), or *rna1-1* (NN19-5B, Noguchi *et al.* 1997). Ura^+ transformants plated on synthetic medium lacking uracil were incubated at the indicated temperature.

compared to the value obtained in the presence of EDTA (data not shown).

To further determine the nucleotide-binding ability of Gtr1p, [^{3}H]GTP was mixed with an increasing amount of cold GTP, GDP, or ATP, and was then incubated with *E. coli*-produced, GST-fused Gtr1p. After incubation at 30° for 30 min, the radioactivity coprecipitated with the glutathione Sepharose-4B beads was quantified using a liquid scintillation counter. In the presence of GTP, the amount of [^{3}H]GTP bound to Gtr1p was greatly reduced (Figure 3A). On the other hand, ATP did not prevent [^{3}H]GTP from binding to Gtr1p, even at the higher concentrations. Compared with the effect of ATP, GDP significantly inhibited the binding of [^{3}H]GTP to Gtr1p, indicating that GDP bound to Gtr1p in a manner that competed with GTP.

The GDP-binding ability of Gtr1p was further confirmed using the Gtr1p-Q65L that may have a defect in GTPase similar to the Q61L mutant of *ras*^{H} (Der *et al.* 1986), which has been reported to bind to both GTP and GDP *in vitro* (Temeles *et al.* 1985; Klebe *et al.* 1995a). Indeed, *E. coli*-produced, His6-fused Gtr1p-Q65L bound to [^{3}H]GTP and to [^{3}H]GDP (Figure 3B). Taken together, these results lead us to conclude that Gtr1p binds to either GTP or GDP, like a normal G protein would. We could not, however, biochemically assay the GTPase activity of Gtr1p *in vitro*. Hence, Gtr1p was referred to as a putative G protein.

Gtr1p interacts with itself: Gtr1p has a long C-terminal tail (from 200 to 310 aa) outside the nucleotide-binding domains (Figure 1A). It contains a large number of leucine residues and is predicted to also contain a coiled-coil motif (Lupas *et al.* 1991; Lupas 1996), both of which are thought to be involved in protein-protein interaction. Using the two-hybrid method, we investigated whether or not Gtr1p could interact with itself through its C terminus (Fields and Song 1989). The wild-type (WT) and the C-terminal-deleted Gtr1p (Gtr1-12Δp) were fused to either the *GAL4 TAD* or the *GAL4 DBD*, and then introduced into the Y190 strain. Considerable β-galactosidase activity was observed when *TAD-GTR1* was coexpressed with *DBD-GTR1* (Table 3). When the wild-type *GTR1* was coexpressed with the C-terminal-deleted mutant *gtr1-12*Δ, however, no significant β-galactosidase activity was detected (Table 3). Similar to wild-type Gtr1p, Gtr1-12Δp was produced in *S. cerevisiae* to a significant degree (Figure 4A, compare lanes 1 and 6). Thus, the failure to detect β-galactosidase activity when the wild-type Gtr1p and Gtr1-12Δp were coexpressed did not result from a low expression of Gtr1-12Δp, implying that the C-terminal tail of Gtr1p is required for self-interaction of Gtr1p.

To further confirm that Gtr1p forms a complex with itself, the *HA*- and *GST-GTR1* clones were cointroduced into the NBW5ΔGTR1 (*gtr1-1*Δ) strain. Crude extracts were prepared, mixed with glutathione Sepharose-4B beads, and the beads were then pelleted. The resulting

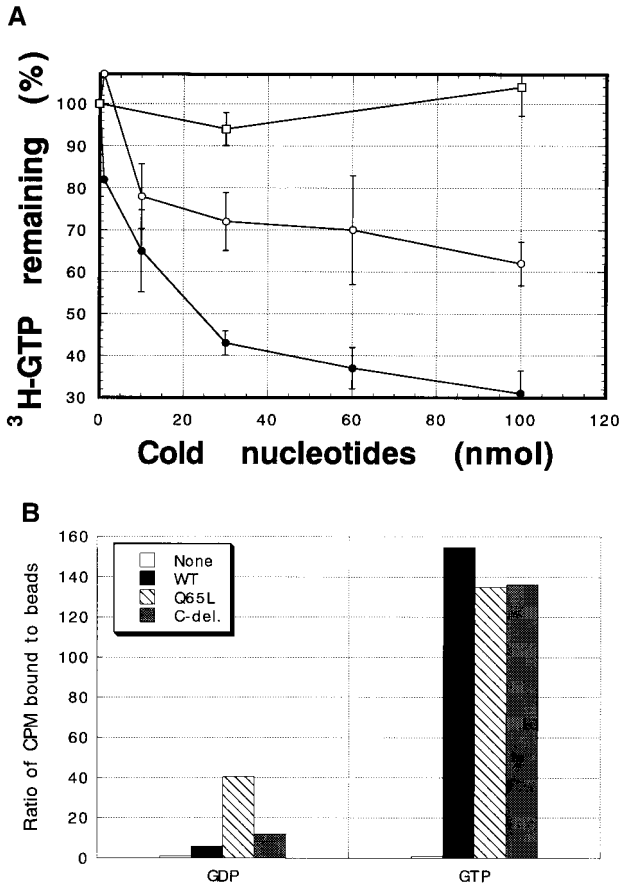


Figure 3.—Nucleotide-binding ability of Gtr1p. (A) *E. coli*-produced GST-Gtr1p (4 nmol), which were bound to the glutathione Sepharose-4B beads, were mixed in S buffer with [^{3}H]GTP (33.8 Ci/mmol:150 pmol) in the presence of the indicated amount of cold GTP (●), GDP (○), or ATP (□). After incubation at 30° for 30 min, the beads were spun down, washed, and the radioactivity coprecipitated with the beads was quantified using a liquid scintillation counter. The vertical axis indicates the ratio (percentage) of the CPM bound to Gtr1p in the presence of the indicated amount of cold nucleotides, on the basis of CPM bound to Gtr1p without cold nucleotides. (B) A total of 0.8 nmol of the *E. coli*-produced His6-Gtr1p proteins [wild type (Gtr1p), Q65L (Gtr1-13p), and C-del. (Gtr1-12Δp)] that were bound to Ni-NTA-Agarose (Qiagen) and, as a control, the Ni-NTA-Agarose beads alone were mixed in S buffer with either [^{3}H]GDP or GTP (33.8 Ci/mmol:150 pmol). After incubation at 30° for 30 min, the beads were spun down, washed, and the radioactivity coprecipitated with the beads was quantified using a liquid scintillation counter. The vertical axis indicates the ratio of the count per minute (CPM) bound to His6-Gtr1p and its derivatives on the basis of CPM bound to beads alone (None).

precipitates were analyzed for the presence of HA-Gtr1p by immunoblotting using the mAb to HA. As shown in Figure 4B, lane 4, HA-Gtr1p was coprecipitated with GST-Gtr1p.

Self-interaction of Gtr1p depends upon the bound nucleotide state: The amount of HA-Gtr1p coprecipitated with GST-Gtr1p was reduced in the presence of an increasing amount of EDTA (Figure 4B, lanes 5 and

TABLE 3

Self interaction of Gtr1p

GAL4TAD-fused *GAL4DBD*-fused	None	*GTR1* (WT)	*gtr1-12*Δ (C-del.)	*gtr1-11* (S20L)	*gtr1-16* (S20N)	*gtr1-13* (Q65L)	*gtr1-15* (S15V)
GTR1 (WT)	0.61 ± 0.29	27 ± 3.5	0.77 ± 0.31	0.82 ± 0.14	0.70 ± 0.081	2.5 ± 0.75	108 ± 20
*gtr1-12*Δ (C-del.)	1.4 ± 0.25	1.8 ± 0.51	2.5 ± 0.62	ND	ND	ND	ND
gtr1-11 (S20L)	1.1 ± 0.35	1.3 ± 0.19	ND	1.5 ± 0.21	ND	ND	ND
gtr1-16 (S20N)	0.59 ± 0.16	0.52 ± 0.13	ND	ND	0.55 ± 0.16	0.55 ± 0.34	ND
gtr1-13 (Q65L)	1.5 ± 0.14	99 ± 5.3	ND	ND	1.2 ± 0.68	24 ± 7.2	ND
gtr1-15 (S15V)	0.34 ± 0.17	70 ± 20	ND	ND	ND	ND	174 ± 38

One unit was calculated as 1 nmol *o*-nitrophenyl-β-d-galactopyranoside cleaved per minute per milligram of protein at 28°. The values are average ± SE for three independent transformants.

ND, not determined.

6). Because EDTA releases the nucleotides from Ran (Bischoff *et al.* 1995; Richards *et al.* 1995), this finding suggests that Gtr1p's interaction with itself is dependent upon the nucleotide bound. It is also possible, however, that the self-interaction of Gtr1p was inhibited by EDTA because of the instability of nucleotide-free Gtr1p, as was the case with nucleotide-free Ran (Klebe *et al.* 1995b).

To determine whether self-interaction of Gtr1p is dependent upon the bound nucleotide state, putative GDP- or GTP-bound mutants of Gtr1p were fused with either the *GAL4 TAD* or the *GAL4 DBD*. As shown in Table 3, when *TAD-gtr1-Q65L* was coexpressed with *DBD-gtr1-Q65L*, a strong transactivation of β-galactosidase was observed, but there was no transactivation of *lacZ* when *TAD-gtr1-S20L* was coexpressed with *DBD-gtr1-S20L* or when *TAD-gtr1-S20N* was coexpressed with *DBD-gtr1-S20N*. The immunoblotting analysis of yeast lysates revealed that the mutant Gtr1p proteins were present at levels similar to those of wild-type Gtr1p (Figure 4A). These findings, therefore, imply that the ability of Gtr1p to interact with itself is dependent upon the bound nucleotide state. This interpretation prompted us to ask whether the lack of interaction shown by the C-terminal deletion was caused by the loss of nucleotide-binding ability. To address this issue, His6-tagged Gtr1-12Δp, and as controls, His6-fused wild-type and Gtr1p-Q65L, were expressed in *E. coli*. Purified His6-fused Gtr1p was then examined for nucleotide-binding ability. Similar to the wild-type and Gtr1p-Q65L, Gtr1-12Δp bound efficiently to GTP (Figure 3B), revealing that the inability of Gtr1-12Δp to interact with itself was not caused by the loss of nucleotide-binding ability.

Gtr1p belongs to a novel family of small G proteins: RagA and RagB have been reported to be mammalian homologues of Gtr1p (Schurmann *et al.* 1995). By homology search, we found a novel *S. cerevisiae* homologue of *GTR1*, designated *GTR2* (GenBank accession no. AB015239, Figure 5A), and a *Caenorhabditis elegans* gene possessing an open reading frame homologous to Gtr1p (GenBank accession no. Z49912/CET24F1-1, Figure 5A).

Gtr1p and its homologues do not have a lipid modification site that is characteristic of Ras (Bun-ya *et al.* 1992; Boguski and McCormick 1993). In this regard, Gtr1p is similar to Ran/Gsp1p (Drivas *et al.* 1990; Bischoff and Ponstingl 1991; Belhumeur *et al.* 1993). Phylogenetic tree analysis (Higgins *et al.* 1992) revealed, however, that Gtr1p belonged to a family different from the Ran/Gsp1p family (Figure 5B). Indeed, all the Gtr1p homologues so far found possess histidine in the third conserved domain, the sequence of which is HKXD (Figure 5A), unlike the NKXD sequence found in the Ran/Ras family (Valencia *et al.* 1991). These findings indicate that Gtr1p defines a novel family of G proteins.

***GTR2* rescues *gtr1-11*, but not *gtr1-1*Δ:** To investigate potential functional interactions between Gtr1p and Gtr2p, we examined the consequence of overexpressing Gtr2p. We amplified *GTR2* by PCR using the primers shown in Figure 6A and inserted it into a multicopy vector, YEplac195, under its own promoter. Overproduction of Gtr2p partially rescued the cold sensitivity of the *gtr1-11* strain, but not that of the *gtr1-1*Δ strain (Figure 6B). Thus, Gtr1p cannot be replaced by Gtr2p.

We also examined the consequences of Gtr2p loss by creating a null allele in the diploid N43 strain (Figure 6A). Cultures of the N43ΔGTR2 strain were sporulated and subjected to tetrad analysis. Most tetrads showed a ratio of viable to nonviable segregants of 4:0, demonstrating that *GTR2* was not essential for survival (data not shown). The disruption of *GTR2* in a haploid strain caused the yeast to become cold sensitive (Figure 6C). On the basis of this finding, we constructed a double-null disruptant (Δ*gtr1* Δ*gtr2*) of *GTR1* and *GTR2* that grew well at 30°, the permissive temperature for each mutant, implying that Δ*gtr1* was not synthetically lethal with Δ*gtr2*.

Self-interaction of Gtr2p requires Gtr1p: Because Gtr1p interacted with itself, we asked whether Gtr2p also interacts with itself. *GTR2* was fused to either the *GST*- or *HA*-tag, and the constructs were introduced into the *gtr2-1*Δ strain. As a control, both *GST*- and *HA-GTR1*

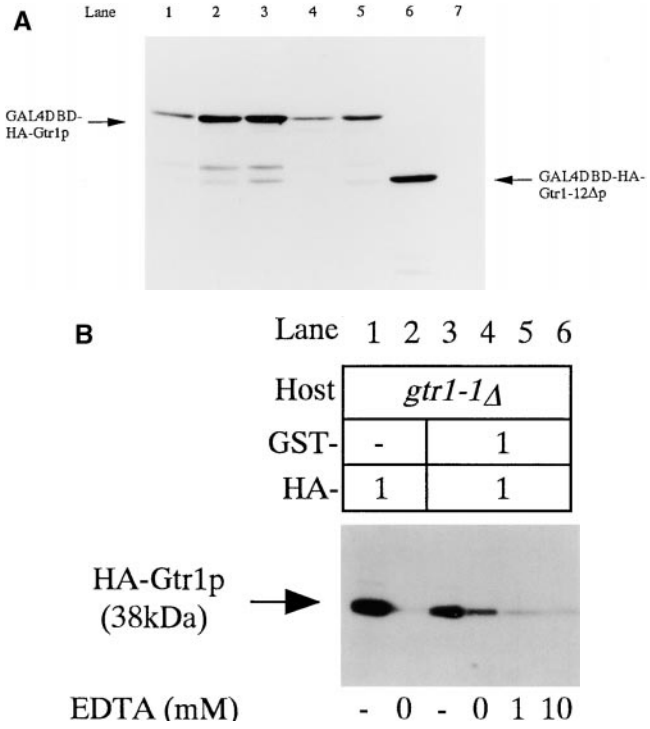


Figure 4.—Self-interaction of Gtr1p. (A) Expression of GAL4-DBD-HA-fused, wild-type and mutated *GTR1* clones in *S. cerevisiae*. Plasmid, pL44 (wild type, lane 1), pL208 (*gtr1-15*, lane 2), pL209 (*gtr1-16*, lane 3), pL66 (*gtr1-11*, lane 4), pL106 (*gtr1-13*, lane 5), and pL87 (*gtr1-12*Δ, lane 6) were introduced into the strain Y190. The Trp^+ transformants selected were cultured in YPD medium at 30° until the early log phase, and then crude extracts were prepared and analyzed by immunoblotting using the mAb to HA-tag. Lane 7 was the extract from nontransfected Y190 cells. (B) Coprecipitation of GST- and HA-fused Gtr1p. The two plasmids, pL38 carrying *GST-GTR1* and pL80 carrying *HA-GTR1* (lanes 3–6), as well as GST vector pEG-KG and pL80 as controls (lanes 1 and 2), were coexpressed in the strain *gtr1-1*Δ. (NBW5ΔGTR1). Prepared crude extracts were mixed with glutathione Sepharose-4B beads, and they were pulled down either in the absence (0, lanes 2 and 4) or presence of EDTA (1.0 and 10 mm, lanes 5 and 6). Proteins bound to beads were analyzed by immunoblotting with the mAb to HA-tag. Lanes 1 and 3, total crude extract.

were coexpressed in the *gtr1-1*Δ strain. Transformants were selected in synthetic medium lacking uracil and tryptophan, crude extracts were prepared, GST-fused Gtr2p or Gtr1p was pulled down with glutathione Sepharose-4B beads, and the resultant precipitates were analyzed for the presence of HA-Gtr2p or Gtr1p by immunoblotting using the mAb to HA. As shown in Figure 7A, HA-Gtr2p was coprecipitated with GST-Gtr2p (lanes 5–8), similar to the case of Gtr1p (lanes 1–4).

Because *GTR2* rescued the cold sensitivity of the *gtr1-11* strain, we examined whether Gtr2p interacts with Gtr1p. *GST*-fused *GTR2* or *GTR1* was coexpressed with *HA*-fused *GTR1* or *GTR2* in the NBW5ΔGTR1/ΔGTR2 (*gtr1-1*Δ *gtr2-1*Δ) strain, as indicated in Figure 7B. From the crude extracts of Ura^+ and Trp^+ transformants, GST-fused proteins were pulled down with glutathione Sepharose-4B beads. The resultant precipitates were analyzed for the presence of HA-fused proteins by immunoblotting using the mAb to HA. As shown in Figure 7B, Gtr1p was coprecipitated with Gtr2p, indicating that Gtr2p forms complexes with Gtr1p. Interestingly, HA-Gtr2p was not coprecipitated with GST-Gtr2p in the NBW5ΔGTR1/ΔGTR2 strain (Figure 7C, lane 4). Hence, self-interaction of Gtr2p requires Gtr1p.

Disruption of *GTR2* suppresses *prp20*: The requirement of Gtr1p for the self-interaction of Gtr2p suggested that the function of Gtr2p is dependent upon Gtr1p. This notion is consistent with the finding that the overexpression of Gtr2p rescued *gtr1-11*, but not *gtr1-1*Δ. Although we do not know whether Gtr2p interacts with GTP-Gtr1p, Gtr2p could be an effector downstream of Gtr1p. If so, *prp20* might be suppressed by a defect in Gtr2p, because *gtr1-11*, which encodes a presumed inactive form of Gtr1p, suppresses *prp20* and *rna1-1*. To address this issue, *GTR2* was disrupted in the strain HS203 (*prp20-1*), as described in materials and methods. Resultant HS203ΔGTR2 (*prp20-1 gtr2-1*Δ) and HS203 strains were then transfected with wild-type *gtr1-S20N* or *gtr1-Q65L* carried on the multicopy vectors. Ura^+ transformants were plated on synthetic medium lacking uracil and were then incubated at 26°, 30°, or 31°, which are the permissive, semipermissive, or nonpermissive temperatures, respectively, for *prp20-1*.

As expected, loss of *GTR2* suppressed *prp20-1* (Figure 8A). Moreover, loss of *GTR2* suppressed the inhibitory effects of *gtr1-Q65L* (Figure 8A, 30°). This finding is consistent with the idea that Gtr2p functions downstream of Gtr1p (see Figure 10). However, the disruption of *GTR1* did not suppress *prp20-1* (Figure 8B). Furthermore, the double disruption of *GTR1* and *GTR2* did not increase the ability to rescue the temperature sensitivity of *prp20-1* (Figure 8B). Hence, it is unlikely that Gtr1p and Gtr2p have parallel functions in the Ran GTPase cycle (see Figure 10).

No effect on nucleocytoplasmic transport: The strain prp20/2c (*prp20-1*) has a defect in mRNA splicing and export (Forrester *et al.* 1992). To clarify the effect of Gtr2p on the Ran/Gsp1p GTPase cycle, we examined whether the mRNA export defect in *prp20* can be rescued by overproduction of Gtr1p-S20L or disruption of *GTR2*. We also examined whether overproduction of Gtr1p-Q65L exacerbates the defect of mRNA export. When cultures of the prp20/2c (*prp20-1*) strain were incubated at 37°, the nuclear accumulation of $poly(A)^+$ RNA was observed (data not shown). Such a defect in mRNA export was not rescued by overexpression of Gtr1p-S20L or disruption of *GTR2*. Furthermore, Gtr1p-Q65L did not show any effect on mRNA export. Consistent with the finding that the *GTR1*/*GTR2* pathway has no effect on mRNA export, both Δ*gtr1* and Δ*gtr2* do not show any defect in either NLS-dependent nuclear protein import or nuclear export signal (NES)-depen-

A

```
Gtr2p          MS------------------------------LEATDSKAMVLLMGVRRCGKSSICKVVFHNMQPLDTLYLESTSNPSLEHFSTLID-------------
Gtr1p          MSSNN-----------------------------------RKKLLLMGRSGSGKSSMRSIIFSNYSAFDTRRLGATI-----------------DVEHSHLR
ORF (C.e.)     MSS-------------------------------------KRKVLLMGKSGSGKTSMRSIIFANYIARDCSRLGPTI-----------------EVEHAHVK
RagA (H.s.)    M---------------------------------PNTAMKKKVLLMGKSGSGKTSMRSIIFANYIARDTRRLGATI-----------------DVEHSHVR
RagB (H.s.)    MEESDSEKTTEKENLGPRMDPPLGEPEGSLGWVLPNTAMKKKVLLMGKSGSGKTSMRSIIFANYIARDTRRLGATI------------NTKTFDVEHSHVR
Consensus                                              XOOOOGXXGXGKS/T
               *                                    .  .****    **.*.  ..* *    *.  * .*.

Gtr2p          -LAVMELP-----GQLNYFEPSYDSER--LFKSVGALVYVIDSQD-EYINAITNLAMIIEYAYKVNPSINIEVLIHKVDGLSED-----FKV-DAQRDIMQ
Gtr1p          FLGNMTLNLWDCGGQDVFMENYFTKQKDHIFQMVQVLIHVFDVESTEVLKDIEIFAKALKQLRKYSPDAKIFVLLHKMDLVQLDKREELFQ------IMMK
ORF (C.e.)     FLGNMVLHLWDCGGQESFMENFLVSQKDQIFKNVQVLIYVFDVESREFEKDLRYYQSCLEALLQNSPNAQVFCLIHKMDLIEEDKREETFKRREADVLRYS
RagA (H.s.)    FLGNLVLNLWDCGGQDTFMENYFTSQRDNIFRNVEVLIYVFDVESRELEKDMHYYQSCLEAILQNSPDAKIFCLVHKMDLVQEDQRDLIFKEREEDLRRLS
RagB (H.s.)    FLGNLVLNLWDCGGQDTFMENYFTSQRDNIFRNVEVLIYVFDVESRELEKDMHYYQSCLEAILQNSPDAKIFCLVHKMDLVQEDQRDLIFKEREEDLRRLS
Consensus            OJOODXAGJX                                                    OOOOHKMD
                 *. . *      **  . *     ..  .*. *  *. *.* . *  . .      .    . .*  ..  *.**.* .  *     *.

Gtr2p          RTGEELLELGLDGVQVSFYLTSIFDHSIYEAFSRIVQKLIPELSFLENMLDNLIQHSKIEKAFLFDVNSKIYVSTDSNPVDIQMYEVCSEFIDVTIDLFDL
Gtr1p          NLSETSSEFGFPNLIG--FPTSIWDESLYKAWSQIVCSLIPNMSNHQSNLKKFKEIMNALEIILFE-RTTFLVICSSNGENSNENHDSSDNNNVLLDPKRF
ORF (C.e.)     ELAATPLQNERTNAVCQCFKSSIWDETLYKAWSAIVYHLVPNVGTMEDKLKKFGAILDADEVILFE-RATFLVIA---------HAVLREHK----DPHRF
RagA (H.s.)    R----PLE-------CACFRTSIWDETLYKAWSSIVYQLIPNVQQLEMNLRNFAQIIEADEVLLFE-RATFLVIS---------HYQCKEQR----DVHRF
RagB (H.s.)    R----PLE-------CSCFRTSIWDETLYKAWSSIVYQLIPNVQQLEMNLRNFAEIIEADEVLLFE-RATFLVIS---------HYQCKEQR----DAHRF
                      .          . .** *...* * * **  *.*..   .  * ..         .**.  . . *               .      *   .

Gtr2p          YKAP-VLRNSQKSSDKDNVINPRNELQNVSQLANGVIIYLRQMIRGLALVAIIRPNGTDMESCLTVADYNIDIFKKGLEDIWANARASQAKNSIEDDV
Gtr1p          EKISNIMKNFKQSCTKLK-----SGFKTLILNNN---IYVSELSSNMVCFIVLKDMNIPQELVLENIKKAKEFFQ-----------------------
ORF (C.e.)     EKVSNIIKQFKLSCTKMG-----SKLDSIQVRNSQFSAFIDSFTQNTYVMIVIPDGNASAPMITMNVKNARKVFEA-IET---------------KS-
RagA (H.s.)    EKISNIIKQFKLSCSKLA-----ASFQSMEVRNSNFAAFIDIFTSNTYVMVVMSDPSIPSAATLINIRNARKHFEK-LERV------DGPKHSLLMR-
RagB (H.s.)    EKISNIIKQFKLSCSKLA-----ASFQSMEVRNSNFAAFIDIFTSNTYVMVVVSDPSIPSAATLINIRNARKHFEK-LERV------DGPKQCLLMR-
                * . .... . *  *         .  .    .    ..           ..                    *
```

B

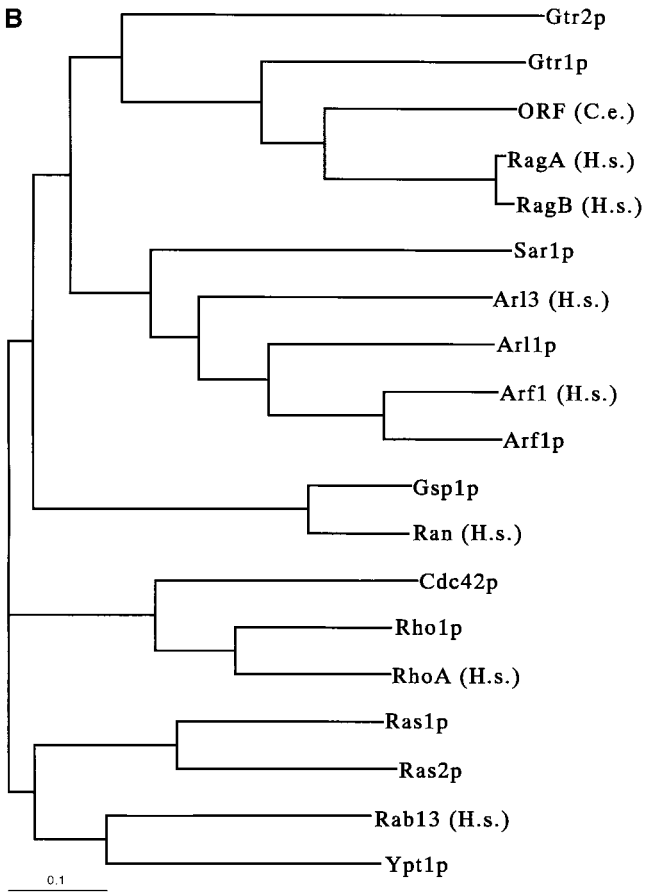


Figure 5.—Gtr1p constitutes a novel family of small GTPases. (A) Alignment of amino acid sequence of Gtr1p and its homologues. ORF (C. e.) is the *C. elegans* genome (GenBank accession number CET24F1-1). Asterisks and periods indicate amino acid residues that are identical and chemically conserved, respectively. Boxes indicate the amino acid sequences conserved among small GTPases. (B) Phylogenetic tree analysis of Gtr1p and other small GTPases. Using the whole amino acid sequence of listed GTPases, the evolutionary relationship was calculated by the CLUSTAL W multiple alignment program (Higgins *et al.* 1992). The bar of 0.1 indicates 10% divergence figures between each pair of sequences. X, any amino acid; O, hydrophobic amino acid; J, hydrophilic amino acid.

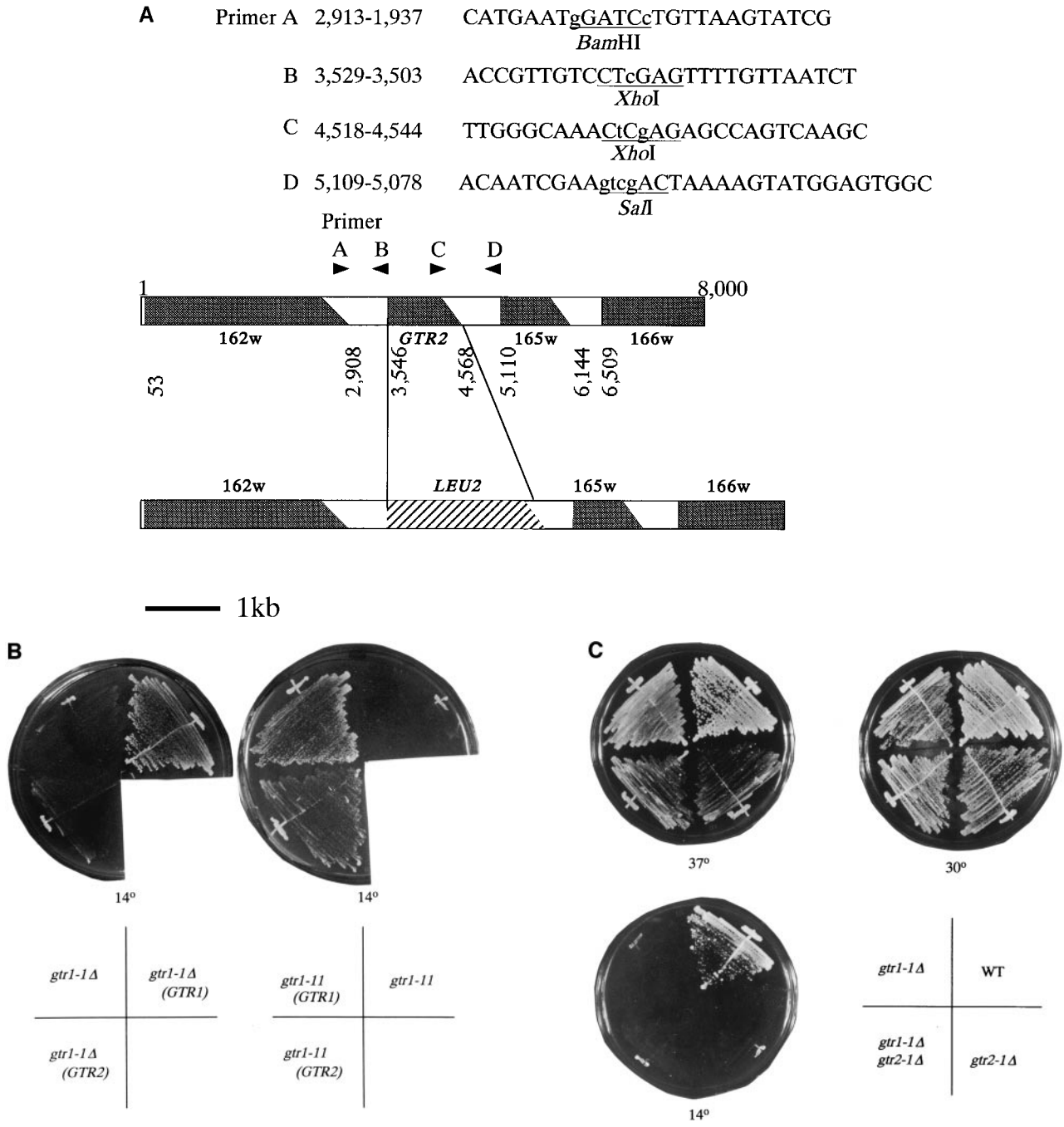


Figure 6.—Function of the *GTR2* gene. (A) Genomic map of the *GTR2* gene. The region of chromosome VII's right arm containing the *GTR2* gene is shown. The number on the map corresponds to the number of the nucleotide. For instance, the *GTR2* gene begins at 3546 bp and ends at 4568 bp. Primers in which small letters indicate the nucleotides that were changed to make an indicated restriction enzyme site were used to disrupt the *GTR2* gene and to clone the *GTR2* gene. Restriction enzyme sites indicated on primers were created to engineer the recombinant *GTR2*. (B) Overexpression of Gtr2p rescues *gtr1-11*, but not Δ*gtr1*. *GTR2* carried on a multicopy YEplac195 pL130, and, as a control, *GTR1* carried on the same vector, pL63, were introduced into the *gtr1-11* strain (NN7-3B) and the *gtr1-1*Δ strain (NBW5ΔGTR1) as indicated. Ura$^+$ transformants were plated on synthetic medium lacking uracil and incubated at 14°. (C) Δ*gtr2* is cold sensitive, but not synthetically lethal with Δ*gtr1*. WT (NBW5), *gtr1-1*Δ (NBW5ΔGTR1), *gtr2-1*Δ (NBW5ΔGTR2), and *gtr1-1*Δ *gtr2-1*Δ (NBW5ΔGTR1/ΔGTR2) strains were plated on YPD medium and then incubated at 14°, 30°, or 37°, as indicated.

dent protein export, even at the nonpermissive temperature (data not shown). Hence, we presumed that Gtr2p regulates the Ran/Gsp1p GTPase cycle through unknown pathways other than the nucleus/cytosol exchange of macromolecules.

Localization of Gtr2p: Gtr1p has been reported to be localized within both the nucleus and the cytoplasm (Nakashima *et al.* 1996). To determine the localization of Gtr2p, T7-tagged Gtr2p was expressed in the NBW5ΔGTR2 and NBW5ΔGTR1/ΔGTR2 strains. As

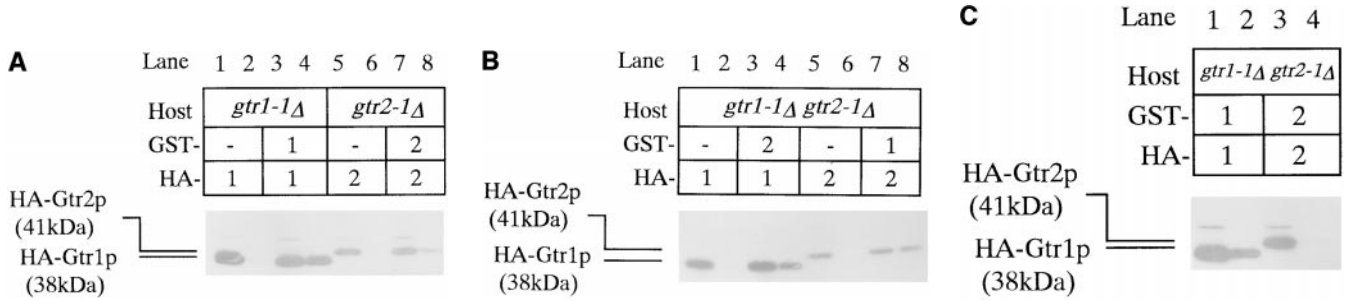


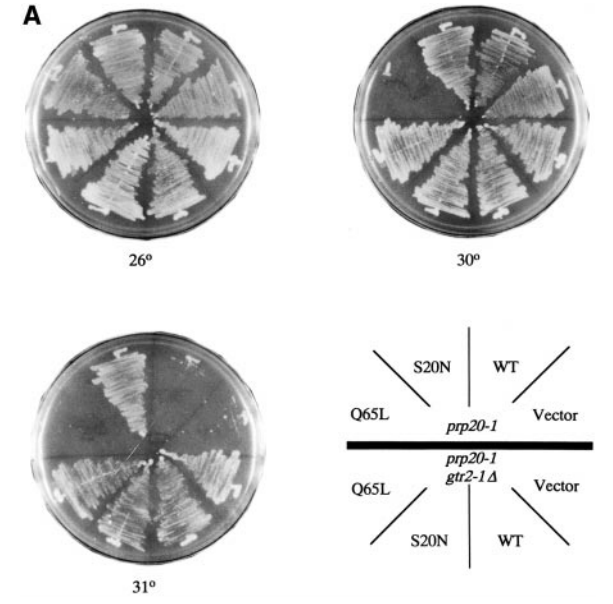
Figure 7.—Gtr2p binds with itself in the presence of Gtr1p. (A) *HA-GTR1* (pL80) and *GST-GTR1* (pL38, lanes 3 and 4) and, as a control, *HA-GTR1* and *GST* alone (pEG-KG, lanes 1 and 2) were coexpressed in the strain *gtr1-1*Δ (NBW5ΔGTR1). *HA-GTR2* (pL154) and *GST-GTR2* (pL155, lanes 7 and 8) and, as a control, *HA-GTR2* and *GST* alone (lanes 5 and 6) were similarly coexpressed in the strain *gtr2-1*Δ (NBW5ΔGTR2) as indicated. Prepared crude extracts were mixed with the glutathione Sepharose-4B beads and pulled down. Proteins bound to beads (lanes 2, 4, 6, and 8) and, as a control, the total crude extracts (lanes 1, 3, 5, and 7), were analyzed by immunoblotting with the mAb to HA-tag. (B) *HA*-fused *GTR1* (pL80, HA-1) or *GTR2* (pL154, HA-2) was coexpressed with *GST-fused GTR2* (pL155, GST-2) or *GTR1* (pL38, GST-1), or with *GST* alone (pEG-KG)(−) in the strain *gtr1-1*Δ *gtr2-1*Δ (NBW5ΔGTR1/ΔGTR2), as indicated. Prepared crude extracts were mixed with the glutathione Sepharose-4B beads and pulled down. Total crude extracts (lanes 1, 3, 5, and 7) and proteins bound to beads (lanes 2, 4, 6, and 8) were analyzed by immunoblotting with the mAb to HA-tag. (C) Either *HA-GTR1* (pL80) and *GST-GTR1* (pL38, lanes 1 and 2), or else *HA-GTR2* (pL154) and *GST-GTR2* (pL155, lanes 3 and 4), were coexpressed in the strain *gtr1-1*Δ *gtr2-1*Δ (NBW5ΔGTR1/ΔGTR2). Crude extracts were mixed with the glutathione Sepharose-4B beads and pulled down. Total crude extracts (lanes 1 and 3) and proteins bound to beads (lanes 2 and 4) were analyzed by immunoblotting with the mAb to HA-tag.

a control, T7-tagged Gtr1p was expressed in the NBW5ΔGTR1 and NBW5ΔGTR1/ΔGTR2 strains. T7-tagged Gtr1p and Gtr2p rescued the cold sensitivity of Δ*gtr1* and Δ*gtr2* strains, respectively. Gtr1p was distributed throughout both the cytoplasm and the nucleus, as reported previously (Nakashima *et al.* 1996). On the other hand, Gtr2p was concentrated in the nucleus, while some of Gtr2p was also localized in the cytoplasm (Figure 9). The staining pattern of Gtr2p was the same in Δ*gtr1* Δ*gtr2* cells (data not shown). Because Gtr1p and Gtr2p were produced in similar amounts, these results indicate that Gtr2p has a tendency to accumulate in the nucleus.

DISCUSSION

Gtr1p has been reported to bind only to GTP, so it was thought to be a putative G protein (Bun-ya *et al.* 1992). In this study, the fact that Gtr1p is indeed a guanine nucleotide-binding protein was shown by direct biochemical experiments. Specifically, *E. coli*-produced, wild-type Gtr1p binds either to GTP or GDP, although its ability to bind to GDP is low when compared with GTP. We also showed that Gtr1p-Q65L, presumed by analogy with Ras to be locked in the GTP-bound form, binds with GTP and GDP. Furthermore, the *in vitro* activity of Gtr1p depends upon its bound nucleotide state as follows. First, *gtr1-S20N* and *gtr1-S20L*, which are putative GDP-bound mutants of *GTR1*, suppress both *prp20* and *rna1*, but *gtr1-Q65L*, which is a putative GTP-bound mutant, does not. Rather, overexpression of *gtr1-Q65L* is inhibitory for both *prp20* and *rna1* cells. Second, Gtr1p interacts with itself in a manner dependent upon the bound nucleotide state. Both wild-type and Gtr1p-Q65L interact with each other, but Gtr1p-S20N and Gtr1p-S20L do not. Thus, GTP-, but not GDP-bound Gtr1p, is suggested to form a complex with itself. We suspect that Gtr1p-Q65L accumulates as a GTP-bound form in cells because of its insensitivity to the GTPase activation enzyme. Taken together, these results suggest that Gtr1p is a G protein, although we could not demonstrate its GTPase activity. Phylogenetic tree analysis indicates that Gtr1p belongs to a novel G protein family that is composed of Gtr1p, Gtr2p, the mammalian homologues RagA and RagB, and an uncharacterized *C. elegans* open reading frame (GenBank accession no. Z49912/CET24F1-1). The protein encoded by the *C. elegans* genome is 46.5% homologous to Gtr1p and 65.0% homologous to human RagA, including chemically conserved amino acid residues.

Gtr2p is homologous to Gtr1p. As for Gtr1p, Gtr2p interacts with itself. However, self-interaction of Gtr2p requires Gtr1p. This finding indicates that Gtr1p forms a complex with Gtr2p. Given that Gtr1p and Gtr2p form a complex, it is noteworthy that they exhibit some of the same genetic interactions: disruptions of *GTR2* and the *gtr1-11* mutation both suppress *prp20-1*. We assume that Gtr2p is an effector downstream of Gtr1p, as shown in Figure 10. The fact that the loss of *GTR2* suppresses *prp20-1* is consistent with the presumption that *gtr1-11* encodes a putative GDP-bound, inactive mutant of Gtr1p. The inactive G protein could not turn on the downstream cascade; this resulted in the same effect as the loss of a downstream effector. Consistent with this interpretation, Gtr1p-Q65L, a putative GTP-bound and, therefore, active form of Gtr1p, inhibits the growth of *prp20-1* and *rna1-1* strains. The fact that the growth inhibitory effect of Gtr1p-Q65L on *prp20-1* is abolished by the disruption of *GTR2* is consistent with the notion that Gtr2p is a downstream effector of Gtr1p. These



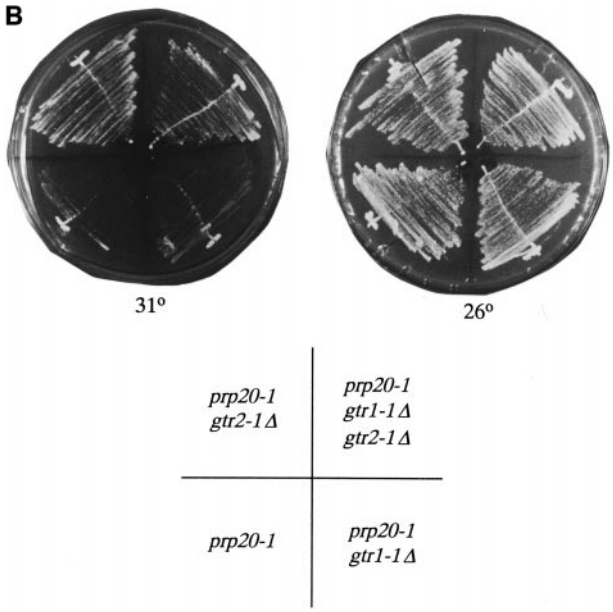


Figure 8.—Effect of the gene disruptions *gtr2-1*Δ and *gtr1-1*Δ on *prp20-1*. (A) YEplac195 plasmids carrying the indicated mutated and wild-type *GTR1* genes and, as a control, the vector alone were introduced into the strains HS203 (*prp20-1*) and HS203ΔGTR2(*prp20-1 gtr2-1*Δ), respectively. Ura$^+$ transformants plated on synthetic medium lacking uracil were incubated at the indicated temperature. (B) Strains HS203 (*prp20-1*), HS203ΔGTR1 (*prp20-1 gtr1-1*Δ), HS203ΔGTR2 (*prp20-1 gtr2-1*Δ), and HS203ΔGTR1,2 (*prp20-1 gtr1-1*Δ *gtr2-1*Δ) were plated on a YPD medium plate and incubated at 31° and 26°, as indicated.

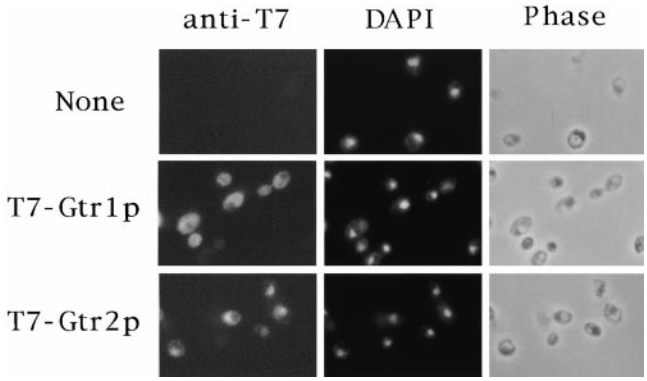


Figure 9.—Nuclear localization of Gtr2p. T7-tagged *GTR1* and *GTR2* were introduced into strains *gtr1-1*Δ and *gtr2-1*Δ, respectively. Ura$^+$ transformants selected in synthetic medium lacking uracil were fixed and stained with the mAb to T7- tag as described (Nakashima *et al.* 1996). DNA was stained with 4′,6-daimidino-2-phenylindole (DAPI).

results suggest that GTP-Gtr1p has a negative effect on both Prp20p and Rna1p. Taking account of the fact that Prp20p and Rna1p are the GDP/GTP-exchanging and GTPase-activating factors of Gsp1p, respectively, it would seem that Gtr1p may negatively regulate the Ran/Gsp1p cycle through Gtr2p (Figure 10).

The fact that the disruption of *GTR1* does not suppress *prp20-1*, however, suggests that an inhibitory function of Gtr2p on the Ran/Gsp1p cycle is also activated by some factor other than Gtr1p (Figure 10, X). This finding also indicates that Gtr1p-S20L dominantly abolishes the negative effect of Gtr2p on the Ran/Gsp1p cycle. In this regard, both GTP- and GDP-bound Gtr1p may make a complex with Gtr2p. It has been reported recently that the Rho family members Cdc42 and Rac2 form homodimers in the GTP-bound state, and that one of the GTP-bound proteins stimulates the GTP hydrolysis of the other protein (Zhang and Zheng 1998). This way, the GDP-Cdc42/GTP-Cdc42 dimer is produced as a transient state. We presume that Gtr1p-S20L forms a complex with GTP-Gtr2p and inhibits the GTPase activity of Gtr2p, although we do not know for sure that Gtr2p is a G protein.

The finding that the nucleus/cytosol exchange of macromolecules is not affected by Gtr1p-S20L, Gtr1p-Q65L, or Δ*gtr2* suggests that the unknown pathways of

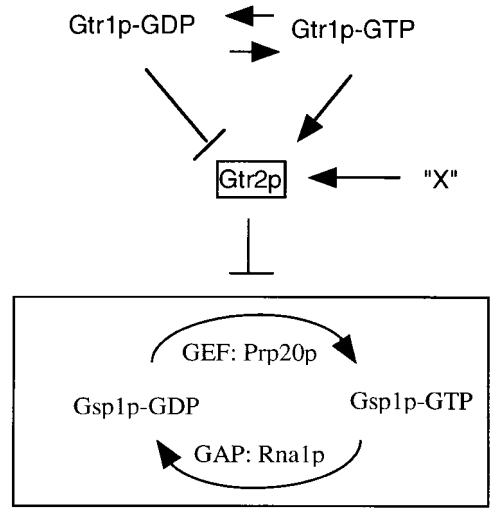


Figure 10.—Interaction of the Gtr1p/Gtr2p cascade with the Gsp1p GTPase cycle.

Ran/Gsp1p other than the nucleus/cytosol exchange of macromolecules are regulated by the Gtr1p/Gtr2p pathway. Dis3p and RanBPM have previously been reported to interact with Gsp1p and Ran. Dis3p is localized in the nucleolus and is suggested to be required for ribosomal RNA processing (Mitchell *et al.* 1997; Shiomi *et al.* 1998). On the other hand, RanBPM is localized in the centrosome, which suggests that it may be involved in microtubule aster formation (Nakamura *et al.* 1998). Although Gtr1p is distributed in both the cytoplasm and the nucleus, Gtr2p seems to be accumulated in the nucleus. We previously found that the human Gtr1p homologue RagA changes its cellular localization depending upon the bound nucleotide state. An interesting question is whether the nuclear localization of Gtr2p is also dependent upon the bound nucleotide state to regulate the Ran/Gsp1p cycle.

We thank M. Funakoshi (Kyushu University) for plasmid YEplac112-XA; H. Sumimoto (Kyushu University) for helpful discussion; and C. Nowak, S. Malak, C. Villa-Braslavsky, J. Kuhlmann, and A. Wittinghofer (Max Planck Institut, Dortmund, Germany) for their help in analyzing *E. coli*-produced Gtr1p at the beginning of this work. This work was supported by Grants-in-Aid for Specially Promoted Research and by Human Frontier Science Program. The English used in this manuscript was revised by K. Miller (Royal English Language Centre, Fukuoka, Japan).

LITERATURE CITED

Aebi, M., M. W. Clark, U. Vijayraghavan and J. Abelson, 1990 A yeast mutant, *PRP20*, altered in mRNA metabolism and maintenance of the nuclear structure, is defective in a gene homologous to the human gene *RCC1* which is involved in the control of chromosome condensation. Mol. Gen. Genet. **224:** 72–80.

Amberg, D. C., A. L. Goldstein and C. N. Cole, 1992 Isolation and characterization of *RAT1*: an essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA. Genes Dev. **6:** 1173–1189.

Avis, J. M., and P. Clarke, 1996 Ran, a GTPase involved in nuclear processes: its regulators and effectors. J. Cell Sci. **109:** 2423–2427.

Belhumeur, P., A. Lee, R. Tam, T. D'Paolo, N. Fortin *et al.*, 1993 *GSP1* and *GSP2*, genetic suppressors of *prp20-1* mutant in *Saccharomyces cerevisiae*: GTP-binding proteins involved in the maintenance of nuclear organization. Mol. Cell. Biol. **13:** 2152–2161.

Bischoff, F. R., and H. Ponstingl, 1991 Mitotic regulator protein RCC1 is complexed with a nuclear ras-related polypeptide. Proc. Natl. Acad. Sci. USA **88:** 10830–10834.

Bischoff, F. R., H. Krebber, E. Smirnova, W. Dong and H. Ponstingl, 1995 Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. EMBO J. **14:** 705–715.

Boguski, M., and F. McCormick, 1993 Proteins regulating Ras and its relatives. Nature **366:** 643–653.

Broach, J. R., J. N. Strathern and J. B. Hicks, 1979 Transformation in yeast: development of a hybrid cloning vector and isolation of the *CAN1* gene. Gene **8:** 121–133.

Bun-ya, M., S. Harashima and Y. Oshima, 1992 Putative GTP-binding protein, Gtr1, associated with the function of the Pho84 inorganic phosphate transporter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:** 2958–2966.

Chi, N. C., E. J. H. Adam, G. D. Visser and S. A. Adam, 1996 RanBP1 stabilizes the interaction of Ran with p97 in nuclear protein import. J. Cell Biol. **135:** 559–569.

Clark, K. L., and G. F. Sprague, Jr., 1989 Yeast pheromone response pathway: characterization of a suppressor that restores mating to receptorless mutants. Mol. Cell. Biol. **9:** 2682–2694.

Coutavas, E., M. Ren, J. D. Oppenheim, P. D'Eustachio and M. G. Rush, 1993 Characterization of proteins that interact with the cell-cycle regulatory protein Ran/TC4. Nature **366:** 585–587.

Dasso, M., T. Seki, Y. Azuma, T. Ohba and T. Nishimoto, 1994 A mutant form of the Ran/TC4 protein disrupts nuclear function in Xenopus laevis egg extracts by inhibiting the RCC1 protein, a regulator of chromosome condensation. EMBO J. **13:** 5732–5744.

Der, C. J., T. Finkel and G. M. Cooper, 1986 Biological and biochemical properties of human rasH genes mutated at codon 61. Cell **44:** 167–176.

Dingwall, C. D., S. Kandels-Lewis and B. Seraphin, 1995 A family of Ran binding proteins that includes nucleoporins. Proc. Natl. Acad. Sci. USA **92:** 7525–7529.

Drivas, G. T., A. Shih, E. Coutavas, M. G. Rush and P. D'Eustachio, 1990 Characterization of four novel RAS-related genes expressed in a human teratocarcinoma cell line. Mol. Cell. Biol. **10:** 1793–1798.

Durfee, T., K. Becherer, P.-L. Chen, S.-H. Yeh, Y. Yang *et al.*, 1993 The retinoblastoma protein associated with the protein phosphatase type 1 catalytic subunit. Genes Dev. **7:** 555–569.

Fields, S., and O.-K. Song, 1989 A novel genetic system to direct protein-protein interaction. Nature **340:** 245–246.

Forrester, W., F. Stutz, M. Rosbash and M. Wickens, 1992 Defects in mRNA 3′-end formation, transcription initiation, and mRNA transport associated with the yeast mutation *prp20*: possible coupling of mRNA processing and chromatin structure. Genes Dev. **6:** 1914–1926.

Funakoshi, M., H. Sikder, H. Ebina, K. Irie, K. Sugimoto *et al.*, 1997 Xenopus cyclin A1 can associate with Cdc28 in budding yeast, causing cell-cycle arrest with an abnormal distribution of nuclear DNA. Genes Cells **2:** 329–343.

Gietz, R. D., and A. Sugino, 1998 New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:** 527–534.

Görlich, D., 1998 Transport into and out of the cell nucleus. EMBO J. **17:** 2721–2727.

Görlich, D., and I. W. Mattaj, 1996 Nucleocytoplasmic transport. Science **271:** 1513–1518.

Görlich, D., M. Dabrowski, F. R. Bischoff, U. Kutay, P. Bork *et al.*, 1997 A novel class of RanGTP binding proteins. J. Cell Biol. **138:** 65–80.

Guan, K. L., and J. E. Dixon, 1991 Eukariotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. **192:** 262–267.

Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi and S. J. Elledge, 1993 The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75:** 805–816.

Hayashi, N., H. Seino, K. Irie, M. Watanabe, K. L. Clark *et al.*, 1996 Genetical interaction of *DED1* encoding a putative ATP-dependent RNA helicase with *SRM1* encoding a mammalian RCC1 homologue in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **253:** 149–156.

Higgins, D. G., A. J. Bleasby and R. Fuchs, 1992 CLUSTAL V: improved software for multiple sequence alignment. Comput. Appl. Biosci. **8:** 189–191.

Hill, J. E., K. A. G. Donald and D. E. Griffiths, 1991 DMSO-enhanced whole cell yeast transformation. Nucleic Acids Res. **19:** 5791.

Hirose, E., N. Nakashima, T. Sekiguchi and T. Nishimoto, 1998 RagA is a functional homologue of *S. cerevisiae* Gtr1p involved in the Ran/Gsp1-GTPase pathway. J. Cell Sci. **111:** 11–21.

Hopper, A. K., F. Banks and V. Evangelidis, 1978 A yeast mutant which accumulates precursor tRNAs. Cell **19:** 211–219.

Hutchison, H. T., L. H. Hartwell and C. S. McLaughlin, 1969 Temperature-sensitive yeast mutant defective in ribonucleic acid production. J. Bacteriol. **99:** 807–814.

Ichihara, Y., and Y. Kurosawa, 1993 Construction of new T vectors for direct cloning of PCR products. Gene **130:** 153–154.

Kadowaki, T., D. Goldfarb, L. M. Spitz, A. M. Tartakoff and M. Ohno, 1993 Regulation of RNA processing and transport by a nuclear guanine nucleotide release protein and members of the Ras superfamily. EMBO J. **12:** 2929–2937.

Kaiser, C., S. Michealis and A. Mitchell, 1994 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kandels-Lewis, S., and B. Seraphin, 1993 Role of U6 snRNA in 5′ splice selection. Science **262:** 2035–2039.

Klebe, C., F. R. Bischoff, H. Ponstingl and A. Wittinghofer, 1995a Interaction of the nuclear GTP-binding protein Ran with its regulatory proteins RCC1 and RanGAP1. Biochemistry **34:** 639–647.

Klebe, C., H. Prinz, A. Wittinghofer and R. S. Goody, 1995b The kinetic mechanism of Ran-nucleotide exchange catalyzed by RCC1. Biochemistry **34:** 12543–12552.

Li, Y., J. Kang and M. S. Horwitz, 1997 Interaction of an adenovirus 14.7-kilodalton protein inhibitor of tumor necrosis factor alpha cytolysis with a new member of the GTPase superfamily of signal transducers. J. Virol. **71:** 1576–1582.

Lupas, A., 1996 Prediction and analysis of coiled-coil structures. Methods Enzymol. **266:** 513–525.

Lupas, A., M. Van Dyke and J. Stock, 1991 Predicting coiled coils from protein sequences. Science **252:** 1162–1164.

Matsuzaki, H., R. Nakajima, J. Nishiyama, H. Araki and Y. Oshima, 1990 Chromosome engineering in *Saccharomyces cerevisiae* by using a site-specific recombination system of yeast plasmid. J. Bacteriol. **172:** 610–618.

Melchior, F., and L. Gerace, 1995 Mechanisms of nuclear protein import. Curr. Opin. Cell Biol. **7:** 310–318.

Melchior, F., T. Guan, N. Yokoyama, T. Nishimoto and L. Gerace, 1995 GTP hydrolysis by Ran occurs at the nuclear pore complex in an early step of protein import. J. Cell Biol. **131:** 571–581.

Mitchell, D. A., T. K. Marshall and R. J. Deschenes, 1993 Vectors for the inducible overexpression of glutathione S-transferase fusion proteins in yeast. Yeast **9:** 715–723.

Mitchell, P., E. Petfalski, A. Shevchenko, M. Mann and D. Tollervey, 1997 The exosome: a conserved eucaryotic RNA processing complex containing multiple 3′-5′ exoribonucleases. Cell **91:** 457–466.

Moore, M. S., and G. Blobel, 1993 The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature **365:** 661–663.

Moore, M., and G. Blobel, 1994 A G protein involved in nucleocytoplasmic transport: the role of Ran. Trends Biochem. Sci. **19:** 211–216.

Mueller, L., V. C. Cordes, F. R. Bischoff and H. Ponstingle, 1998 Human RanBP3, a group of nuclear RanGTP binding proteins. FEBS Lett. **427:** 330–336.

Nakamura, M., H. Masuda, J. Horii, K. Kuma, N. Yokoyama *et al.*, 1998 A novel centrosomal protein, RanBPM, when overexpressed, causes ectopic microtubule nucleation, similar to γ-tubulin. J. Cell Biol. **143:** 1041–1052.

Nakashima, N., N. Hayashi, E. Noguchi and T. Nishimoto, 1996 Putative GTPase GTR1 genetically interacts with RanGTPase cycle in *Saccharomyces cerevisiae*. J. Cell Sci. **109:** 2311–2318.

Nigg, E. A., 1997 Nucleocytoplasmic transport: signals, mechanisms and regulation. Nature **386:** 779–787.

Nishitani, H., M. Ohtubo, K. Yamashita, H. Iida, J. Pines *et al.*, 1991 Loss of RCC1, a nuclear DNA-binding protein, uncouples the completion of DNA replication from the activation of cdc2 protein kinase and mitosis. EMBO J. **10:** 1555–1564.

Noguchi, E., N. Hayashi, Y. Azuma, T. Seki, M. Nakamura *et al.*, 1996 Dis3, implicated in mitotic control, directly binds to Ran and enhances the GEF activity of RCC1. EMBO J. **15:** 5595–5605.

Noguchi, E., N. Hayashi, N. Nakashima and T. Nishimoto, 1997 Yrb2p, Nup2p-related yeast protein, has functional overlap with Rna1p, yeast RanGAP protein. Mol. Cell. Biol. **17:** 2235–2246.

Nuoffer, C., F. Peter and W. E. Balch, 1995 Purification of His6-tagged Rab1 proteins using bacterial and insect cell expression systems. Methods Enzymol. **101:** 3–9.

Oki, M., E. Noguchi, N. Hayashi and T. Nishimoto, 1998 Nuclear protein import, but not mRNA export, is defective in all of the temperature-sensitive mutants of the *Saccharomyces cerevisiae* Ran homologue, Gsp1-GTPase. Mol. Gen. Genet. **257:** 624–634.

Richards, S. A., K. M. Lounsbury and I. G. Macara, 1995 The C terminus of the nuclear RAN/TC4 GTPase stabilizes the GDP-bound state and mediates interactions with RCC1, RAN-GAP, and HTF9A/RANBP1. J. Biol. Chem. **270:** 14405–14411.

Sazer, S., 1996 The search for the primary function of the Ran-GTPase continues. Trends Cell Biol. **6:** 81–85.

Sazer, S., and P. Nurse, 1994 A fission yeast RCC1-related protein is required for the mitosis to interphase transition. EMBO J. **13:** 606–615.

Schlenstedt, G., D. H. Wong, D. M. Koepp and P. A. Silver, 1995 Mutants in a yeast Ran binding protein are defective in nuclear transport. EMBO J. **14:** 5367–5378.

Schurmann, A., A. Brauers, S. Massmann, W. Becker and H-G. Joost, 1995 Cloning of a novel family of mammalian GTP-binding proteins (RagA, $RagB^s$, $RagB^l$) with remote similarity to the Ras-related GTPase. J. Biol. Chem. **270:** 28982–28988.

Seki, T., N. Hayashi and T. Nishimoto, 1996 RCC1 in the Ran pathway. J. Biochem. **120:** 207–214.

Shiomi, T., K. Fukushima, N. Suzuki, N. Nakashima, E. Noguchi *et al.*, 1998 Human Dis3p, which binds to either GTP- or GDP-Ran, complements *Saccharomyces cerevisiae dis3*. J. Biochem. **123:** 883–890.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Taura, T., H. Krebber and P. A. Silver, 1998 A member of the Ran-binding protein family, Yrb2p, is involved in nuclear protein export. Proc. Natl. Acad. Sci. USA **95:** 7427–7432.

Temeles, G. L., J. B. Gibbs, J. S. D'Alonzo, I. S. Sigal and E. M. Scolnick, 1985 Yeast and mammalian *ras* proteins have conserved biochemical properties. Nature **313:** 700–703.

Uchida, S., T. Sekiguchi, H. Nishitani, K. Miyauchi, M. Ohtsubo *et al.*, 1990 Premature chromosome condensation is induced by a point mutation in the hamster *RCC1* gene. Mol. Cell. Biol. **10:** 577–584.

Ullman, K. S., M. A. Powers and D. J. Forbes, 1997 Nuclear export receptors: from importin to exportin. Cell **90:** 967–970.

Valencia, A., P. Chardin, A. Wittinghofer and C. Sander, 1991 The ras protein family: evolutionary tree and role of conserved amino acids. Biochemistry **30:** 4637–4648.

Wong, D., A. H. Corbett, H. M. Kent, M. Stewart and P. A. Silver, 1997 Interaction between the small GTPase Ran/Gsp1p and Ntf2p is required for nuclear transport. Mol. Cell. Biol. **17:** 3755–3767.

Wu, J., J. M. Matunis, D. Kraemer, G. Blobel and E. Coutavas, 1995 Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin A homologous domain, and a leucine-rich region. J. Biol. Chem. **270:** 14209–14213.

Yokoyama, N., N. Hayashi, T. Seki, N. Pante, T. Ohba *et al.*, 1995 A giant nucleopore protein that binds Ran/TC4. Nature **376:** 184–188.

Zhang, B., and Y. Zheng, 1998 Negative regulation of Rho family GTPases Cdc42 and Rac2 by homodimer formation. J. Biol. Chem. **273:** 25728–25733.

Communicating editor: A. G. Hinnebusch