Nuclear protein import, but not mRNA export, is defective in all *Saccharomyces cerevisiae* mutants that produce temperature-sensitive forms of the Ran GTPase homologue Gsp1p

著者	OKI M, NOGUCHI E, HAYASHI N, NISHIMOTO T
journal or publication title	Molecular and General Genetics
volume	257
number	6
page range	624-634
year	1998-04
URL	http://hdl.handle.net/10098/1628

doi: 10.1007/s004380050690

M. Oki · E. Noguchi · N. Hayashi · T. Nishimoto

Nuclear protein import, but not mRNA export, is defective in all *Saccharomyces cerevisiae* mutants that produce temperature-sensitive forms of the Ran GTPase homologue Gsp1p

Abstract A series of ts mutations in the *GSP1* gene of *Saccharomyces cerevisiae* was isolated by error-prone PCR. A total of 25 ts *gsp1* strains was obtained. Each of these mutants showed between one and seven different amino acid alterations. In several of these ts *gsp1* strains, the same amino acid residues in Gsp1p were repeatedly mutated, indicating that our screen for ts *gsp1* mutations was saturating. All of the ts *gsp1* strains isolated had a defect in nuclear protein import, but only 16 of the 25 ts *gsp1* strains had a defect in mRNA export. Thus, Gsp1p is suggested to be directly involved in nuclear protein import, but not in mRNA export. Following release from α -factor arrest, 11 of the ts *gsp1* mutants arrested in G1; the remainder did not show any specific cell-cycle arrest, at 37°C, the nonpermissive temperature. While the mutants that are defective in both mRNA export and protein import have a tendency to arrest in G1, there was no clear correlation between the cell cycle phenotype and the defects in mRNA export and nuclear protein import. Based on this, we assume that Ran/Gsp1p GTPase regulates the cell cycle and the nucleus/cytosol exchange of macromolecules through interactions with effectors that were independent of each other, and are differentially affected by mutation.

Key words Ran · Gsp1 · Nuclear protein import · RNA export

Communicated by C. P. Hollenberg

M. Oki · E. Noguchi · N. Hayashi¹ · T. Nishimoto (✉)
Department of Molecular Biology,
Graduate School of Medical Science,
Kyushu University, Higashi-ku,
Fukuoka 812-8582, Japan
Fax: +81-92-642-6183;
e-mail: nishi@molbiol.med.kyushu-u.ac.jp

Present address:

¹Cancer Research Institute, Kanazawa University,
Kanazawa, Ishikawa 920, Japan

Introduction

The Ran GTPase gene was originally isolated as a member of the Ras superfamily, using a mixed-oligonucleotide probe based on a conserved Ras domain, from a cDNA library constructed with RNA from an undifferentiated human teratocarcinoma, and is designated as TC4 (Drivas et al. 1990). RCC1 was isolated as a human gene that complements a temperature-sensitive (ts) *rcc1*⁻ mutant, tsBN2, of the hamster BHK21 cell line (Kai et al. 1986; Ohtsubo et al. 1987). On the other hand, a protein of 24 kDa that co-purifies with RCC1 from HeLa cells was designated as Ran (Bischoff and Ponstingl 1991a). Subsequently, Bischoff and Ponstingl (1991b) found that Ran is identical to TC4 and that RCC1 is a GDP/GTP exchange factor for the Ran/TC4 GTPase. They have also purified the Ran GTPase activating protein, designated as RanGAP1, which turned out to be a mammalian homologue of *Saccharomyces cerevisiae* Rna1p (Bischoff et al. 1995a, b). *rna1-1*, a ts mutant of the *RNA1* gene was isolated as a mutant that is defective in tRNA splicing (Hopper et al. 1987). Like RanGAP1, RCC1 is conserved in evolution. Temperature-sensitive mutants for *RCC1* homologues have been isolated in studies of diverse aspects of cell biology in yeast species – as mRNA splicing (*prp20*) (Aebi et al. 1990), signal transduction (*srml-1*) (Clark and Sprague 1989) and mRNA export (*mtr1*) (Kadowaki et al. 1993) in *S. cerevisiae*, and as mutants that are defective in cell cycle regulation (*pim1-d1*) in *Schizosaccharomyces pombe* (Matsumoto and Beach 1991; Sazer and Nurse 1994).

Independently, Ran GTPase has been found to be essential for nuclear localization signal (NLS)-dependent nuclear protein import (Moore and Blobel 1993; Melchior et al. 1993). In addition to nuclear protein import, the phenotype of *rcc1*⁻ mutants indicates that Ran GTPase is involved in mRNA export as well. Indeed, the GTP-bound form of Ran GTPase is required for mRNA export (Schlenstedt et al. 1995a; Richards et al. 1997).

Taking all these results into account, it can be argued that the pleiotropic phenotype of *rcc1*⁻ is a consequence of defects in nuclear pore transport function. However, we have previously found that premature activation of p34^{cdc2}/cyclin B, which is induced by loss of RCC1 function in tsBN2 cells, can be prevented by nuclear injection of GTP-Ran (Ohba et al. 1996), indicating that some nucleoplasmic processing step is also dependent on GTP-Ran. Indeed, there is a nuclear Ran-binding protein, Yrb2p (Noguchi et al. 1997), in *S. cerevisiae*, which specifically binds to GTP-Ran. Disruption of the *YRB2* gene does not cause any defect in mRNA export or NLS-dependent nuclear protein import (Noguchi et al. 1997). Therefore, Ran does not seem to be solely required for nucleus/cytosol exchange of macromolecules.

Thus far, several proteins have been found to possess a so-called Ran-binding domain (reviewed by Sazer 1996; Seki et al. 1996). Of these, Yrb1p, disruption of which impairs nuclear import (Schlenstedt et al. 1995b), and RanBP2/NUP358, which is localized at the end of the cytoplasmic filament of nuclear pores (Yokoyama et al. 1995; Wu et al. 1995), are involved in nucleus/cytosol exchange of macromolecules, whereas Yrb2p is not (Noguchi et al. 1997). In order to determine how Ran GTPase controls its downstream functions, we have isolated a series of temperature-sensitive mutants for the *S. cerevisiae* Ran homologue, Gsp1p (Belhumeur et al. 1993).

Materials and methods

Strains and media

All the *S. cerevisiae* strains used in this study are described in Table 1. 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). Selection against Ura⁺ strains was carried out by culturing on solid synthetic media containing 1 mg/ml 5-fluoroorotic acid (5-FOA) (Boeke et al. 1984). The media used for *S. cerevisiae* and bacteria have been described previously (Nishiwaki et al. 1987).

Construction of plasmids

The 1.9-kb *HincII*-*SspI* and the 3.3-kb *NsiI*-*XbaI* fragments of pBTCNR1HB4.1 (Kadowaki et al. 1993), both of which contain the open reading frame (ORF) of Gsp1p, were isolated and, respectively, inserted into the *HincII* site of pRS314, resulting in pF314GSP1, and the *NsiI*/*XbaI* site of pUC28, resulting in pUC28NXCNR1.

Disruption of the *GSP1* gene.

The 1.75-kb *HIS3* fragment of YIp1 (Parent et al. 1985) was isolated and inserted into the *Bam*HI site of pBluescriptIISK(+), resulting in pSKHIS3 β ; the 1.75-kb *SpeI*-*SmaI* fragment containing the *HIS3* ORF was then recovered from pSKHIS3 β and inserted into the *SpeI*/*BalI* site of pUC28NXCNR1, resulting in pUC28CNRHIS3. The *XbaI*-*SphI* fragment of pUC28CNRHIS3 was used to disrupt the *GSP1* locus of the *S. cerevisiae* strain N43 by transformation. Disruption of the *GSP1* gene was confirmed by Southern analysis.

Table 1 Plasmids and yeast strains used in this study

	Relevant markers	Comment/genotype	Source
Plasmids			
pBTCNR1HB4.1		pBluescriptIISK(+) with <i>GSP1</i> / <i>CNR1</i> fragment	Kadowaki et al. (1993)
pF314GSP1	<i>CEN TRP1 GSP1</i>	pRS314 with 1.9-kb <i>GSP1</i> fragment at <i>HincII</i> / <i>SspI</i> site	This study
pF314gsp1 ^{ts}	<i>CEN TRP1 gsp1^{ts}</i>	pRS314 with 1.9-kb <i>gsp1^{ts}</i> fragment at <i>HincII</i> / <i>SspI</i> site	This study
pSKHIS3 β		pBluescriptIISK(+) with 1.75-kb <i>HIS3</i> fragment	This study
pUC28NXCNR1		pUC28 with 3.3-kb <i>GSP1</i> / <i>CNR1</i> fragment at <i>NsiI</i> / <i>XbaI</i> site	This study
pUC28CNRHIS3		pUC28NXCNR1 with 1.75-kb <i>HIS3</i> fragment	This study
pFBI-33C	2 μ m <i>URA3</i>	<i>H2B</i> NLS fused in frame to β -galactosidase	Moreland et al. (1987)
pRSCNR1	<i>GAL 10-H2B::lacZ</i> <i>CEN URA3 GSP1/</i> <i>CNR1</i>	pRS316 with <i>GSP1</i> / <i>CNR1</i> fragment	A. Tartakoff
Ylp351GSP1	<i>LEU2 GSP1</i>	Ylp351 with 1.95-kb <i>GSP1</i> fragment ^a	This study
Ylp351gsp1 ^{ts}	<i>LEU2 gsp1^{ts}</i>	Ylp351 with 1.95-kb <i>GSP1^{ts}</i> fragment ^a	This study
Yeasts			
<i>S. cerevisiae</i> N43		<i>MATa</i> / <i>MATa ade2/ade2 his3/his3 leu2/leu2</i> <i>LYS2/lys2 trp1/trp1 ura3/ura3</i>	Noguchi et al. (1997)
<i>S. cerevisiae</i> N43-6C		<i>MATa gsp1::HIS3 ade2 leu2 trp1 ura3</i> [pRSCNR1]	This study
<i>S. cerevisiae</i> N43-6C-GSP1		<i>MATa Δ gsp1::HIS3::GSP1::LEU2 ade2 leu2</i> <i>trp1 ura3</i>	This study
<i>S. cerevisiae</i> N43-6C-gsp1 ^{ts}		<i>MATa Δ gsp1::HIS3::gsp1^{ts}::LEU2 ade2 leu2</i> <i>trp1 ura3</i>	This study

^a Ylp351 is described by Hill et al. (1986)

Fig. 1A, B Schematic preparation of *ts gsp1* strains. **A** Isolation of mutated *ts gsp1* genes. The *GSP1* gene was amplified under mutagenic PCR conditions as described in Materials and methods, using the indicated primers. The PCR product was then cotransformed into N43-6C together with a gapped plasmid (pF314GSP1) containing homology to both ends of the PCR product, based on the method previously described (Muhlrads et al. 1992). Transformants (Ura^+ Trp^+) were selected for *ts* mutants as described in Materials and methods. *gsp1** indicates the mutagenized *GSP1* gene. **B** Integration of the *ts gsp1* into the chromosomal *GSP1* site. Ylp351*gsp1*^{ts} plasmids were integrated into the *HindIII* site on chromosome XII of *S. cerevisiae* indicated by the X. YLR294C and YLR292C are open reading frames on *S. cerevisiae* chromosome XII

Mutagenesis

The *GSP1* gene was mutagenized by error-prone PCR (Beckman et al. 1985; Leung et al. 1989) as follows. Using as M13-20 and M13 reverse primers (TaKaRa), the *GSP1* gene carried on pF314GSP1 was amplified with 5 units of *Taq* polymerase in PCR buffer (7 mM MgCl_2 , 50 mM KCl, 10 mM TRIS-HCl pH 8.3, 0.01% gelatin, 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, 1 mM dTTP, and 0.5 mM MnCl_2) for 30 cycles (94°C, 1 min; 45°C, 1 min; 72°C, 1 min).

Site-directed mutagenesis was performed on pF314GSP1 using the site-directed mutagenesis system Mutan-K (TaKaRa).

Nuclear protein import assay

S. cerevisiae ts gsp1 strains carrying pFB1-33C were cultured in synthetic medium containing raffinose, but lacking uracil, at 26°C until the early log phase, and then incubated at 37°C in synthetic medium containing galactose. When the growth rate began to fall significantly, cells were fixed and stained as described previously (Noguchi et al. 1997).

mRNA export assay

Cells were cultured at 26°C in YPD medium until early log phase, and then incubated at 37°C. When the growth rate started to drop significantly, cells were fixed and in situ hybridization for mRNA was carried out using biotin-labeled oligo(dT)₅₀ as described previously (Noguchi et al. 1997).

Results

Isolation of *ts* mutations in *GSP1*

The *GSP1* gene was amplified by error-prone PCR under the conditions described in Materials and methods. Amplified DNA fragments were introduced into the *S. cerevisiae* strain N43-6C ($\Delta\text{gsp1}::\text{HIS3}$ [pRSCNR1]) (Table 1), together with the gapped pF314GSP1 plasmid (CEN, *TRP1*, *GSP1*) from which the *GSP1* ORF had been removed by digestion with the restriction enzymes *SpyI* and *BamI*, as described in Fig. 1A. Transfected cells were plated on synthetic medium lacking tryptophan and uracil at 26°C. About 4000 Trp^+ Ura^+ colonies, some of which now carry the mutated *GSP1* gene on gapped pF314GSP1, appeared and were replated onto synthetic medium plates containing 1 mg/ml of 5-FOA, which selects against the *URA3* plasmid pRSCNR1. After incubation at 26°C for about 3 days, 2093 colonies were recovered. Thus, the survival rate following mutagenesis was 52%. These colonies were replated onto YPD plates, which were subsequently incubated either at 26°C, the permissive temperature, or at 37°C, the nonpermissive temperature. A schematic representation of the mutant-isolation method is shown in Fig. 1A. Finally, 51 Trp^+ colonies were found to be temperature-sensitive for cell proliferation at 37°C. Representative results of temperature sensitivity tests are shown in Fig. 2.

In order to confirm the temperature sensitivity of the *Gsp1p* encoded by the plasmids, the plasmids were iso-

Fig. 2 Colony formation by *ts gsp1* strains. The *ts gsp1* strains were streaked onto two YPD plates as indicated. Plates were incubated either at 26° C (the permissive temperature), or at 37° C (the nonpermissive temperature) for 3 days

lated from the *ts Trp⁺* clones and retransformed into the N43-6C strain ($\Delta gsp1::HIS3$ [pRSCNR1]). Transfected cells were plated on synthetic medium lacking tryptophan and uracil. After the wild-type plasmid pRSCNR1 had been eliminated by growth in 5-FOA, all of the *Trp⁺* colonies became temperature-sensitive for cell proliferation, indicating that the *GSP1* gene carried by the pF314 plasmids derived from the original *ts Trp⁺* clones indeed encoded a temperature-sensitive form of Gsp1p.

Subsequently, we determined the nucleotide sequence of all 51 of the *ts gsp1* genes and identified the mutation sites. The number of mutated amino acid residues in any

given *ts gsp1* gene ranged from 1 to 7 (Table 2). Several *ts gsp1* genes that were found to contain the same set of amino acid alterations were discarded. Among the *ts gsp1* clones, the same amino acid residues were repeatedly found to be mutated, as shown in Fig. 3. Interestingly, however, amino acid residues in the domains conserved in all small GTPases and essential for nucleotide binding (Boguski and McCormick 1993) were not mutated, with the exception of lysine in the third domain. Mutations in these domains are probably incompatible with survival. Indeed, the mutation of K125R in the third domain alone is lethal (see Discussion).

Table 2 Phenotypes of *ts gsp1* strains

Mutation	Amino acid change	mRNA export ^a	Protein import ^a	Arrest point
<i>gsp1-1757</i>	F28S	– (C)	– (D)	G1
<i>gsp1-1268</i>	A85D	– (C)	– (D)	G1
<i>gsp1-479</i>	I89Y	– (C)	– (D)	G1
<i>gsp1-16</i>	D93E, W165R	+ (A)	– (D)	Non-specific
<i>gsp1-322</i>	H50Q, Y149H	– (D)	– (B)	G1
<i>gsp1-882</i>	L52S, N156D	– (C)	– (D)	G1
<i>gsp1-1547</i>	V103A, K125R	– (C)	– (D)	Non-specific
<i>gsp1-1907</i>	M91V, I117A	– (B)	– (D)	G1
<i>gsp1-245</i>	K14N, K62I, I89F	– (B)	– (D)	G1
<i>gsp1-1178</i>	Y55H, Q86P, E160V	– (B)	– (C)	G1
<i>gsp1-1568</i>	H50L, Y55N, Y100F	– (B)	– (D)	Non-specific
<i>gsp1-1598</i>	R112S, I151F, F178L	– (D)	– (C)	G1
<i>gsp1-1651</i>	W66R, E160G, Q190P	+ (A)	– (C)	Non-specific
<i>gsp1-1819</i>	K14I, K129E, D192E	– (B)	– (D)	Non-specific
<i>gsp1-1894</i>	K14R, T34A, K62E	+ (A)	– (D)	Non-specific
<i>gsp1-1060</i>	V10D, E36G, E60V, N158D	– (D)	– (C)	G1
<i>gsp1-1486</i>	F58S, N84I, K144N, D150G	+ (A)	– (D)	Non-specific
<i>gsp1-640</i>	E9D, F63L, F92L, L121M, K129N	+ (A)	– (C)	Non-specific
<i>gsp1-1582</i>	K73N, I98V, K125R, K129I, L176I	– (B)	– (D)	G1
<i>gsp1-1778</i>	K39M, F58L, N102I, R108K, E193D	+ (A)	– (D)	Non-specific
<i>gsp1-1968</i>	F37Y, H50R, T56S, Y82C, N102D	+ (A)	– (D)	Non-specific
<i>gsp1-1518</i>	E36V, F92Y, N116S, Y199S, D213E, T207A	– (B)	– (D)	Non-specific
<i>gsp1-1260</i>	T56S, E60A, K125R, T137A, A183G, A208G	+ (A)	– (D)	Non-specific
<i>gsp1-1763</i>	L33S, V94A, N116T, T137A, K154M, N173D	+ (A)	– (D)	Non-specific
<i>gsp1-1817</i>	F28Y, T34A, I119T, K143R, Q147R, L195S, Q200H	– (B)	– (D)	Non-specific

^a (A), (B), (C) and (D) indicate the degree of residual activity (as a percentage of the wild-type activity) of *ts* mutants for mRNA export or nuclear protein import, as follows. (A), 80–100% activity (more than 80% of cells showed normal activity); (B), 50–80%; (C),

20–50%; (D), 0–20%. More than 100 cells in the same field were examined. Class A phenotypes are regarded as wild type (+); all others are adjudged to be abnormal (–)

Fig. 3 Mutation sites found in ts *gsp1* genes. The amino acid residues found to be changed in the 51 ts *gsp1* genes examined are shown by the symbol #. For comparison, the amino acid sequences of Spilp and Ran/TC4 GTPase are aligned. The numbers indicate the amino acid positions in Gsp1p. The boxed regions are conserved among all small GTPases. The stars and dots indicate identical and chemically conserved amino acid residues, respectively

Construction of ts *gsp1* strains

In order to characterize further the effect of ts *gsp1* mutations on cell proliferation, the 1.95-kb *Bam*HI-*Xho*I fragment of the ts *gsp1* genes carried by pF314gsp1^{ts} plasmids was removed, and then inserted into the *Bam*HI and *Sal*I sites of the plasmid YIp351 (*LEU2*), resulting in YIp351gsp1^{ts} (*gsp1*^{ts}, *LEU2*) (Fig. 1B). As a control, the wild-type *GSP1* gene was similarly introduced into the YIp351 plasmid (*LEU2*), resulting in YIp351GSP1 (*GSP1*, *LEU2*). YIp351 plasmids containing either wild-type *GSP1* or a ts *gsp1* gene were partially digested with *Hind*III and then introduced into the strain N43-6C (Δ *gsp1::HIS3* [pRSCNR1]), in order to integrate ts *gsp1* genes into the *Hind*III site localized 19 bp downstream of the 3' end of *GSP1* ORF (Fig. 1B). *Leu*⁺ *Ura*⁺ transformants were selected on synthetic medium plates lacking leucine and uracil, and then replated on synthetic medium containing 5-FOA to eliminate the wild-type plasmid pRSCNR1. *Leu*⁺ colonies were replated onto YPD plates at 26°C or 37°C to determine their temperature sensitivities. The entire procedure is shown schematically in Fig. 1B.

Some of the ts *gsp1* genes derived from the original ts *Trp*⁺ colonies were not ts for cell proliferation following the integration of mutated genes into the *GSP1* locus of the *S. cerevisiae* strain N43-6C. A total of 25 *S. cerevisiae* N43-6C-gsp1^{ts} strains possessing a ts mutation in the *GSP1* gene were obtained (Table 2).

Three of the 25 ts *gsp1* strains had a single amino acid alteration. The rest all contained several amino acid changes in the *GSP1* gene. In order to confirm the correlation between the amino acid change in Gsp1p and the ts character, we introduced the amino acid changes

found in ts *gsp1* strains that contained either single or double mutations into the wild-type *GSP1* ORF of plasmid pF314GSP1, by using site-directed mutagenesis. Upon introduction of the resulting mutated pF314GSP1 into the strain N43-6C, it was found that all of the amino acid changes found in those ts *gsp1* genes that had a single mutation caused yeast to become temperature-sensitive for cell proliferation. On the other hand, neither of the two amino acid changes found in the ts *gsp1* genes possessing double mutations conferred the ts character on yeast. Both of the amino acid changes, therefore, were necessary to cause yeast to become temperature-sensitive in cases where ts *gsp1* strains possessed double mutations.

Characterization of ts *gsp1* strains

In order to characterize the ts *gsp1* mutations in liquid cultures, the growth curve of each ts *gsp1* strains was determined. Representative results are shown in Fig. 4. Most ts mutants showed a clear growth defect even in liquid cultures. But some mutants, for example *gsp1-1518*, showed considerable growth at 37°C, the non-permissive temperature, although they eventually arrested (data not shown). At the times indicated by the arrows in Fig. 4 when the growth rate of ts *gsp1* strains started to drop significantly, the mutants' capacities for mRNA export were determined. Similarly, the ability to import proteins into the nucleus was determined in synthetic medium.

Nuclear protein import

Hydrolysis of GTP-Ran is essential for nuclear protein import (Moore and Blobel 1993; Melchior et al. 1993). We therefore investigated the abilities of ts *gsp1* strains to import proteins bearing a nuclear localization signal into the nucleus. To this end, the plasmid pFB1-33C (*URA3*), which encodes β -galactosidase fused in frame with the NLS from histone H2B at a site downstream of

Fig. 4 Growth curves for *ts gsp1* strains. Overnight cultures of *GSP1* and *gsp1^{ts}* strains were diluted into YPD medium and grown for 4 h at 26°C. Half of each culture was then shifted to 37°C (filled circles) and the other half was incubated further at 26°C (open circles). At the indicated times, OD_{660nm} values were measured. At the times indicated by the arrows (4 h for *ts gsp1-16*, *ts gsp1-322*, *ts gsp1-479*, *ts gsp1-1763* and *ts gsp1-1778*; 10 h for *ts gsp1-1817*; and 11 h for *ts gsp1-1486* and *ts gsp1-1518*), cells were sampled to determine the localization of mRNA

the *GAL10* promoter (Moreland et al. 1987), was introduced into the *ts gsp1* strains. Transfected cells were plated on synthetic medium lacking uracil. Ura⁺ transformants were cultured to early log phase at 26°C in synthetic medium containing raffinose, but lacking

uracil, and then incubated at 37°C, the nonpermissive temperature, in synthetic medium containing galactose. When the growth rate of *ts gsp1* strains (Ura⁺) began to fall significantly, as shown in Fig. 4, cells were stained with anti- β -galactosidase antibodies. Representative results are shown in Fig. 5. In our wild-type *S. cerevisiae* strain N43-6C, nuclear protein import was sometimes slow at 26°C, probably due to the lower temperature and the genetic background of our strain. Therefore, the capacity of the *ts gsp1* strains for nuclear protein import was determined at 37°C and compared with that of wild-type strains at the same temperature.

In most of the *ts gsp1* strains, NLS-dependent nuclear protein import was profoundly retarded. In Table 2, we

Fig. 5 Nuclear protein import in *ts gsp1* strains. Wild-type and *ts gsp1-479* strains carrying pFB1-33C were cultured in synthetic medium containing 2% raffinose, but lacking uracil, at 26°C until early log phase, and then incubated at 37°C in synthetic medium containing 2% galactose. When the growth rate began to fall significantly (see Fig. 4), cells were fixed and treated with an anti- β -galactosidase antibody raised in rabbit, followed by Texas red-conjugated goat anti-rabbit antibody (β -gal). Cellular DNA was stained with Hoechst 33342 (Hoechst). Cells in the same field were observed by fluorescence and phase contrast (Phase) microscopy

list the ability of each *ts* mutant to import proteins into the nucleus relative to that of wild-type strain. There were no *ts gsp1* strains which showed a normal nuclear protein import at 37°C, the nonpermissive temperature (Table 2).

mRNA export

Defects in mRNA export are very common in *S. cerevisiae prp20*⁻ mutants and have also been reported in *rna1-1* cells (reviewed by Sazer 1996; Seki et al. 1996). We therefore investigated the ability of *ts gsp1* strains to export mRNA from the nucleus to the cytoplasm.

ts gsp1 cells were cultured in liquid YPD medium at 26°C until early log phase, and then half of each culture was incubated further at 37°C. At the times indicated by the arrows in Fig. 4, cells were fixed and processed for in situ hybridization with biotin-labeled oligo-dT. As controls, cells kept at 26°C were fixed and processed similarly. After hybridization, cells were stained with FITC-avidin and then the nucleus was counter-stained with Hoechst 33342 in PBS buffer. Representative results are shown in Fig. 6. As in the case of nuclear protein import, mRNA export was sometime retarded at 26°C, even in the wild-type strain, as shown in Fig. 6A. At 37°C, however, mRNA was efficiently exported in wild-type cells and in some *ts gsp1* strains which showed

no defect in mRNA export, while other *ts* mutants had no ability to export mRNA (Fig. 6B).

Nine out of the 25 *ts gsp1* strains had no defect in mRNA export (Table 2). In the remainder, the capacity for mRNA export varied. The ability of each *ts* mutant to export mRNA is listed in Table 2 in comparison with that of wild-type strain.

Cell cycle analysis

In order to investigate the relationship between the Ran/Gsp1p GTPase cycle and cell cycle regulation, cultures of *ts gsp1* strains were treated with α -factor at 26°C. Following release from α -factor arrest, half of each culture was incubated at 37°C, the nonpermissive temperature, and the other half was cultured at 26°C, the permissive temperature. The progression of the cell cycle was monitored by FACS analysis (Fig. 7).

Of the *ts gsp1* mutants, 11, all of which have a defect in mRNA export, did not initiate a new cell cycle following release from α -factor arrest at 37°C, while they did enter a new cell cycle at 26°C, the permissive temperature. At the nonpermissive temperature, these mutants were arrested at G1. The remaining mutants accumulated as cells possessing either G1 or G2 DNA

Fig. 6A, B mRNA export in *ts gsp1* strains. The strains *ts gsp1-16* and *ts gsp1-322* and, as a control, the wild-type strain N43-6C-GSP1 were cultured in YPD medium at 26°C until the early log phase. Cultures were then divided in half and one half was further incubated at 26°C (A), while the other half was transferred to 37°C (B). At the times indicated in Fig. 4, when the growth rate started to drop, cells were fixed and hybridized with biotin-labeled oligo(dT)₅₀, followed by incubation with FITC-avidin. Cellular DNA was stained with Hoechst 33342 (Hoechst). FITC and Hoechst fluorescence of the same fields of cells are shown, together with the corresponding phase-contrast views

Fig. 7 Cell cycle analysis of *ts gsp1* strains. *ts gsp1* strains and wild-type strains were cultured in YPD medium at 26°C until an OD_{660nm} of 0.1 and then incubated in the presence of 10 µg/ml of α -factor for 2 h at 26°C. Subsequently, fresh α -factor (20 µg/ml final concentration) was added and the cells were incubated at 26°C for another 2 h. After the α -factor had been washed out with distilled water, half of cells were incubated in YPD medium at 37°C, and the other half at 26°C. At the indicated times, cells were sampled and processed for FACS analysis. *Top panel* Wild-type cells. *Middle panel* *ts gsp1* strains showing G1 arrest. *Bottom panel* *ts gsp1* strains showing non-specific cell cycle arrest

content at 37°C, showing no clear cell cycle-specific arrest. Two representative examples of G1 and non-specific cell cycle arrests are shown in Fig. 7.

Most of the *ts gsp1* strains showed an aberrantly enlarged shape. Representative examples were shown in Fig. 8. The strains *ts gsp1-1757* and *ts gsp1-1778*, respectively, arrest in G1 and in a non-specific manner (Table 2). Hence, the aberrant shape of *ts gsp1* strains does not seem to be correlated with their cell cycle phenotype.

Discussion

In addition to *ts gsp1* genes containing a single mutation, we isolated several other *ts gsp1* genes in which the number of mutation sites ranges from 2 to 7. Both of

the amino acid changes found in double mutations are necessary to make yeast temperature sensitive. Thus, we can assume that all of the amino acid changes found in other *ts gsp1* strains with multiple mutations are necessary for the temperature-sensitive growth phenotype. In this regard, it is noteworthy that the amino acid change of K125R in *ts gsp1-1547* is also present in two other mutants, *ts gsp1-1582* and *ts gsp1-1260*, which, in addition to K125R, possess 5 and 6 amino acid changes, respectively. The concerted effects of these mutation sites may correspond to the effect of the amino acid change V103A in *ts gsp1-1547*. In the case of the K125R mutation, an additional mutation is essential to make yeast temperature-sensitive for cell proliferation, since the *GSP1* plasmid containing the K125R mutation alone is lethal for *S. cerevisiae* (M. Oki, in preparation).

Fig. 8 Cellular morphology of *ts gsp1* cells. *ts gsp1-1757* and *ts gsp1-1778* and, as a control, wild-type strains were cultured at 26°C and photographed under phase-contrast optics. Bar 25 μ m

Among the *ts gsp1* genes obtained, some amino acids are repeatedly changed and several *ts gsp1* genes were found to contain the same set of amino acid changes. Considering all these results together, we can surmise that our mutagenesis for *ts gsp1* genes is almost saturating.

While nuclear protein import in our wild-type strain was sometimes retarded at 26°C, it was normal at 37°C. Thus, the abilities of *ts gsp1* strains to import proteins into the nucleus were examined and compared with that of wild type strain at 37°C. In this regard, it is not certain that the defect in nuclear protein import of *ts gsp1* strains is directly related to the temperature-sensitive growth defect of these strains. Even so, the finding that all of the *ts gsp1* strains isolated here have a defect in nuclear protein import is remarkable. It is consistent with the previous *in vitro* finding that the Ran GTPase is essential for nuclear protein import (Moore and Blobel 1993; Melchior et al. 1993). In contrast to nuclear protein import, defects in mRNA export are not universal among the *ts gsp1* strains. This is surprising because accumulation of mRNA in the nucleus is frequently observed among *S. cerevisiae prp20⁻* mutants and *rna1-1* (Izaurralde and Mattaj 1995), although these mutants do not have a defect in Gsp1p itself. The *prp20⁻* have a defect in the GDP/GTP exchange factor for Gsp1p and the *rna1-1* has a defect in the GTPase-activating factor for Gsp1p GTPase. Our present results indicate that the Gsp1p is not directly involved in mRNA export, while it is directly required for NLS-dependent nuclear protein import.

By analogy with Ras GTPase, the events downstream of Gsp1p action may depend on the function of effectors for the Gsp1p GTPase. *S. cerevisiae* has been reported to contain a family of Ran/Gsp1p GTPase-binding proteins (Dingwall et al. 1995), the number of which is still increasing (Sazer 1996). The *ts* mutations in the *GSP1* gene described here may affect these effectors differentially. While those *ts gsp1* mutants that are defective in both nuclear protein import and mRNA export show G1 arrest, there are no *ts gsp1* strains which arrest at G2 alone. This is curious, since *ts srp1*, which is a *ts* mutant for importin α and is therefore defective in nuclear

protein import, is arrested at G2 following release from α -factor arrest (Loeb et al. 1995). The mutant Gsp1p probably affects several downstream effectors, in addition to abrogating nuclear protein import. In this context, the finding that there are no *ts gsp1* strains which arrest at G2 alone seems reasonable. The cell proliferation phenotype may be the concerted consequence of abnormal function of several Gsp1p effectors which are affected by a given *ts gsp1* mutation. Obviously, many effectors for Gsp1p GTPase remain to be uncovered. By isolating a suppressor for each *ts gsp1* strain, we should be able to identify the effector(s) of Gsp1p GTPase affected by any particular *ts gsp1* mutation.

During the preparation of this manuscript, Wong et al. (1997) reported the isolation of two *ts gsp1* strains. Their sample of *ts* mutants is too small to allow a comprehensive genetic characterization of the function of Gsp1p GTPase. However, these authors found that their *ts* mutants can be suppressed by overexpression of the *NTF2* gene. We are currently investigating the effect of overexpression of *NTF2* gene on our *ts gsp1* strains.

Acknowledgements We thank Dr. S. Sakaguchi for the anti- β galactosidase antibody, Dr. A. M. Tartakoff for the CNR1 plasmid, Drs. L. I. Davis and P. Silver for NLS-histone H2B, and Drs. H. Quadota and H. Seino for PCR mutagenesis. This work was supported by Grants-in-Aid for Specially Promoted Research and by the HFSP. The text was revised by Miss K. Miller (Royal English Language Centre, Fukuoka, Japan).

References

- Aebi M, Clark MW, Vijayraghavan U, Abelson J (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
- Beckman RA, Mildvan AS, Loeb LA (1985) On the fidelity of DNA replication: manganese mutagenesis *in vitro*. *Biochemistry* 24:5810–5817
- Belhumeur P, Lee A, Tam R, DiPaolo T, Fortin N, Clark MW (1993) *GSP1* and *GSP2*, genetic suppressors of the *prp20-1* mutant in *Saccharomyces cerevisiae*: GTP-binding proteins involved in the maintenance of nuclear organization. *Mol Cell Biol* 13:2152–2161

- Bischoff FR, Ponstingl H (1991a) Mitotic regulator protein RCC1 is complexed with a nuclear ras-related polypeptide. *Proc Natl Acad Sci USA* 88:10830-10834
- Bischoff FR, Ponstingl H (1991b) Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature* 354:80-82
- Bischoff FR, Krebber H, Smirnova E, Dong W, Ponstingl H (1995a) Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. *EMBO J* 14:705-715
- Bischoff FR, Krebber H, Kempf T, Hermes I, Ponstingl H (1995b) Human RanGTPase-activating protein RanGAP1 is a homolog of yeast Rna1p involved in mRNA processing and transport. *Proc Natl Acad Sci USA* 92:1749-1753
- Boeke JD, LaCroute F, Fink GR (1984) A positive selection for mutants lacking oridine-5'-phosphate decarboxylase activity in yeast. *Mol Gen Genet* 197:345-346
- Boguski M S, McCormick F (1993) Proteins regulating Ras and its relatives. *Nature* 366:643-653
- Clark KL, Sprague GF Jr (1989) Yeast pheromone response pathway: characterization of a suppressor that restores mating to receptorless mutants. *Mol Cell Biol* 9:2682-2694
- Dingwall CD, Kandels-Lewis S, Seraphin B (1995) A family of Ran binding proteins that includes nucleoporins. *Proc Natl Acad Sci USA* 92:7525-7529
- Drivas GT, Shih A, Coutavas E, Rush MG, D'Eustachio P (1990) Characterization of four novel RAS-related genes expressed in a human teratocarcinoma cell line. *Mol Cell Biol* 10:1793-1798
- Hill J, Donald K, Griffiths DE (1991) DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Res* 19:5791
- Hill JE, Myers AM, Koerner TJ, Tzagoloff A (1986) Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* 2:163-167
- Hopper AK, Banks F, Evangelidis V (1978) A yeast mutant which accumulates precursor tRNAs. *Cell* 19:211-219
- Izaurrealde E, Mattaj JW (1995) RNA export. *Cell* 81:153-159
- Kadowaki T, Goldfarb D, Spitz LM, Tartakoff AM, Ohno M (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
- Kai R, Ohtsubo M, Sekiguchi M, Nishimoto T (1986) Molecular cloning of a human gene that regulates chromosome condensation and is essential for cell proliferation. *Mol Cell Biol* 6:2027-2032
- Kaiser C, Michaelis S, Mitchell A (1994) *Methods in yeast genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- Leung DW, Chen E, Goeddel DV (1989) A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Techniques* 1:11-15
- Loeb JD, Schlenstedt G, Pellman D, Kornitzer D, Silver PA, Fink GR (1995) The yeast nuclear import receptor is required for mitosis. *Proc Natl Acad Sci USA* 92:7647-7651
- Matsumoto T, Beach D (1991) Premature initiation of mitosis in yeast lacking RCC1 or an interacting GTPase. *Cell* 66:347-360
- Melchior F, Paschal B, Evans J, Gerace L (1993) Inhibition of nuclear protein import by nonhydrolyzable analogs of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J Cell Biol* 123:1649-1659
- Moore MS, Blobel G (1993) The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature* 365:661-663
- Moreland RB, Langevin GL, Singer RH, Garcea RL, Hereford LM (1987) Amino acid sequences that determine the nuclear localization of yeast histone 2B. *Mol Cell Biol* 7:4048-4057
- Muhlrad D, Hunter R, Parker R (1992) A rapid method for localized mutagenesis of yeast genes. *Yeast* 8:79-82
- Nishiwaki K, Hayashi N, Irie S, Chung D-H, Harashima S, Oshima Y (1987) Structure of the yeast *HIS5* gene responsive to general control of amino acid biosynthesis. *Mol Gen Genet* 208:159-167
- Noguchi E, Hayashi N, Nakashima N, Nishimoto T (1997) Yrb2p, a Nup2p-related yeast protein has functional overlap with Rna1p, yeast RanGAP protein. *Mol Cell Biol* 17:2235-2246
- Ohba T, Seki T, Azuma Y, Nishimoto T (1996) Premature chromatin condensation induced by loss of RCC1 is inhibited by GTP- and GTP γ S-Ran, but not GDP-Ran. *J Biol Chem* 271:14665-14667
- Ohtsubo M, Kai R, Furuno N, Sekiguchi T, Sekiguchi M, Hayashida H, Kuma K, Miyata T, Fukushima S, Murotsu T, Matsubara K, Nishimoto T (1987) Isolation and characterization of the active cDNA of the human cell cycle gene(RCC1) involved in the regulation of onset of chromosome condensation. *Genes Dev* 1:585-593
- Parent SA, Fenimore CM, Bostian KA (1985) Vector systems for the expression, analysis and cloning of DNA sequences in *S. cerevisiae*. *Yeast* 1:83-138
- Richards SA, Carey KL, Macara IG (1997) Requirement of guanosine triphosphate-bound Ran for signal-mediated nuclear protein export. *Science* 276: 1842-1844
- Sazer S (1996) The search for the primary function of the Ran GTPase continues. *Trends Cell Biol* 6:81-85
- Sazer S, Nurse P (1994) A fission yeast RCC1-related protein is required for the mitosis to interphase transition. *EMBO J* 13:606-615
- Seki T, Hayashi N, Nishimoto T (1996) RCC1 in the Ran pathway. *J Biochem* 120:207-214
- Schlenstedt G, Saavedra C, Loeb JD, Cole CN, Silver PA (1995a) The GTP-bound form of the yeast Ran/TC4 homologue blocks nuclear protein import and appearance of poly(A)⁺ RNA in the cytoplasm. *Proc Natl Acad Sci USA* 92:225-229
- Schlenstedt G, Wong DH, Koepf DM, Silver PA (1995b) Mutants in a yeast Ran binding protein are defective in nuclear transport. *EMBO J* 14:5367-5378
- Yokoyama N, Hayashi N, Seki T, Pante N, Ohba T, Nishii K, Kuma K, Hayashida T, Miyata T, Aebi U, Fukui M, Nishimoto T (1995) A giant nucleopore protein that binds Ran/TC4. *Nature* 376:184-188
- Wong DH, Corbett AH, Kent HM, Stewart M, Silver PA (1997) Interaction between the small GTPase Ran/Gsp1p and Ntf2p is required for nuclear transport. *Mol Cell Biol* 17:3755-3767
- Wu J, M Matunis J, Kraemer D, Blobel G, Coutavas E (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