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

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

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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 (*srm1-1*) (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 *Hinc*II-*Ssp*I and the 3.3-kb *Nsi*I-*Xba*I 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 *Hinc*II site of pRS314, resulting in pF314GSP1, and the *Nsi*I/*Xba*I site of pUC28, resulting in pUC28NXCNRI.

Disruption of the *GSP1* gene.

The 1.75-kb *HIS3* fragment of YIpI (Parent et al. 1985) was isolated and inserted into the *Bam*HI site of pBluescriptIISK(+), resulting in pSKHIS3β; the 1.75-kb *Spe*I-*Sma*I fragment containing the *HIS3* ORF was then recovered from pSKHIS3β and inserted into the *Spe*I/*Bal*I site of pUC28NXCNR1, resulting in pUC28CNRHIS3. The *Xba*I-*Sph*I 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			
pBTCNRIHB4.1		pBluescriptIISK(+) with *GSP1/CNR1* fragment	Kadowaki et al. (1993)
pF314GSP1	*CEN TRP1 GSP1*	pRS314 with 1.9-kb *GSP1* fragment at *Hinc*II/*Ssp*I site	This study
pF314gsp1^{ts}	*CEN TRP1 gsp1^{ts}*	pRS314 with 1.9-kb *gsp1^{ts}* fragment at *Hinc*II/*Ssp*I site	This study
pSKHIS3β		pBluescriptIISK(+) with 1.75-kb *HIS3* fragment	This study
pUC28NXCNR1		pUC28 with 3.3-kb *GSP1/CNR1* fragment at *Nsi*I/*Xba*I site	This study
pUC28CNRHIS3		pUC28NXCNRI with 1.75-kb *HIS3* fragment	This study
pFB1-33C	2 μm *URA3* *GAL 10-H2B::lacZ*	*H2B NLS* fused in frame to β-galactosidase	Moreland et al. (1987)
pRSCNR1	*CEN URA3 GSP1/CNR1*	pRS316 with *GSP1/CNR1* fragment	A. Tartakoff
Ylp351GSP1	*LEU2 GSP1*	Ylp351 with 1.95-kb *GSP1* fragment[a]	This study
Ylp351gsp1^{ts}	*LEU2 gsp1^{ts}*	Ylp351 with 1.95-kb *GSP1^{ts}* fragment[a]	This study
Yeasts			
S. cerevisiae N43		*MAT***a**/*MAT*α *ade2/ade2 his3/his3 leu2/leu2 LYS2/lys2 trp1/trp1 ura3/ura3*	Noguchi et al. (1997)
S. cerevisiae N43-6C		*MAT***a** *gsp1::HIS3 ade2 leu2 trp1 ura3* [pRSCNR1]	This study
S. cerevisiae N43-6C-GSP1		*MAT***a** Δ *gsp1::HIS3::GSP1::LEU2 ade2 leu2 trp1 ura3*	This stydy
S. cerevisiae N43-6C-gsp1^{ts}		*MAT***a** Δ *gsp1::HIS3::gsp1^{ts}::LEU2 ade2 leu2 trp1 ura3*	This study

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

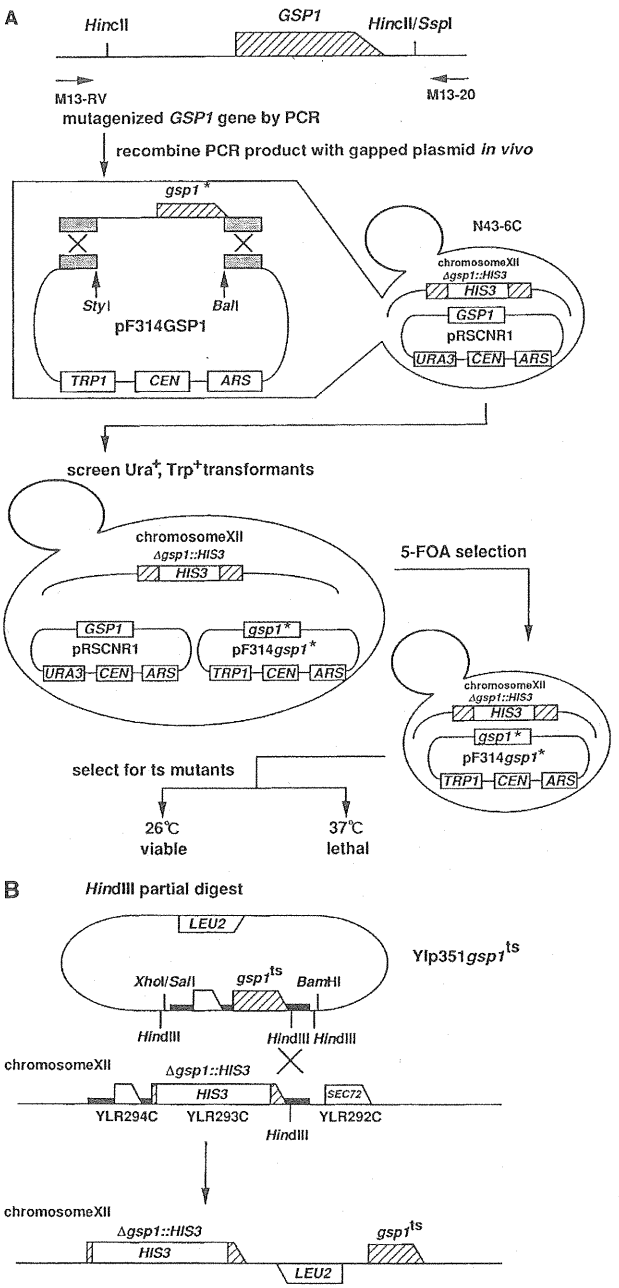


Fig. 1A, B Schematic preparation of ts *gsp1* strains. A Isolation of mutated ts *gsp1* genes. The *GSP1* gene was amplified under mutagenic PCR conditions as described in Materials and methods, using the indicated primers. The PCR product was then cotransformed into N43-6C together with a gapped plasmid (pF314GSP1) containing homology to both ends of the PCR product, based on the method previously described (Muhlrad 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. YIp351gsp1^{ts} plasmids were integrated into the *Hin*dIII 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 (*Δgsp1::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 *Sty*I and *Bal*I, 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-

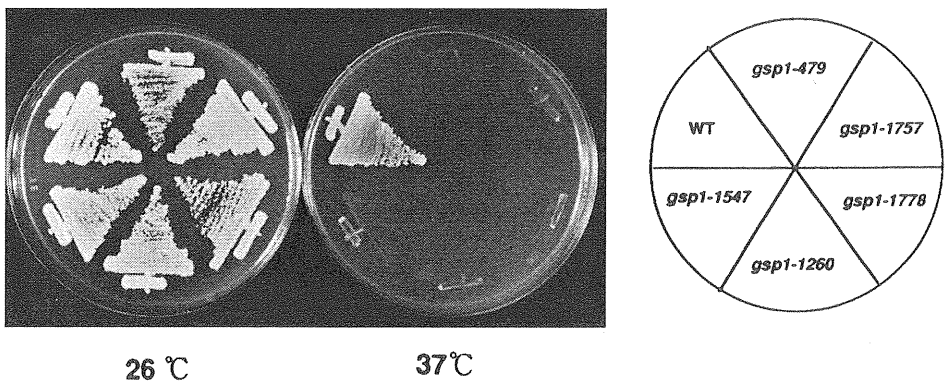


Fig. 2 Colony formation by ts *gsp1* strains. The ts *gsp1* strains were streaked onto two YPD plates as indicated. Plates were incubated either at 26° C (the permissive temperature), or at 37° C (the nonpermissive temperature) for 3 days

lated from the ts Trp^+ clones and retransformed into the N43-6C strain (Δ*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
gsp1-1757	F28S	− (C)	− (D)	G1
gsp1-1268	A85D	− (C)	− (D)	G1
gsp1-479	I89Y	− (C)	− (D)	G1
gsp1-16	D93E, W165R	+ (A)	− (D)	Non-specific
gsp1-322	H50Q, Y149H	− (D)	− (B)	G1
gsp1-882	L52S, N156D	− (C)	− (D)	G1
gsp1-1547	V103A, K125R	− (C)	− (D)	Non-specific
gsp1-1907	M91V,I117A	− (B)	− (D)	G1
gsp1-245	K14N, K621, I89F	− (B)	− (D)	G1
gsp1-1178	Y55H, Q86P, E160V	− (B)	− (C)	G1
gsp1-1568	H50L, Y55N, Y100F	− (B)	− (D)	Non-specific
gsp1-1598	R112S, I151F, F178L	− (D)	− (C)	G1
gsp1-1651	W66R, E160G, Q190P	+ (A)	− (C)	Non-specific
gsp1-1819	K14I, K129E, D192E	− (B)	− (D)	Non-specific
gsp1-1894	K14R, T34A, K62E	+ (A)	− (D)	Non-specific
gsp1-1060	V10D, E36G, E60V, N158D	− (D)	− (C)	G1
gsp1-1486	F58S, N84I, K144N, D150G	+ (A)	− (D)	Non-specific
gsp1-640	E9D, F63L, F92L, L121M, K129N	+ (A)	− (C)	Non-specific
gsp1-1582	K73N, I98V, K125R, K129I, L176I	− (B)	− (D)	G1
gsp1-1778	K39M, F58L, N102I, R108K, E193D	+ (A)	− (D)	Non-specific
gsp1-1968	F37Y, H50R, T56S, Y82C, N102D	+ (A)	− (D)	Non-specific
gsp1-1518	E36V, F92Y, N116S, Y199S, D213E, T207A	− (B)	− (D)	Non-specific
gsp1-1260	T56S, E60A, K125R, T137A, A183G, A208G	+ (A)	− (D)	Non-specific
gsp1-1763	L33S, V94A, N116T, T137A, K154M, N173D	+ (A)	− (D)	Non-specific
gsp1-1817	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 (−)

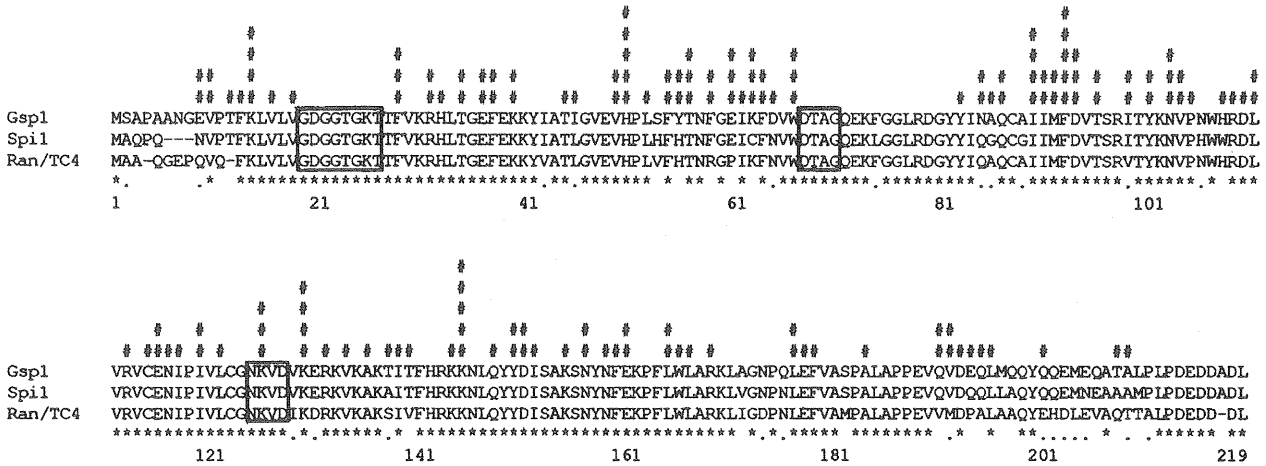


Fig. 3 Mutation sites found in ts *gsp1* genes. The amino acid residues found to be changed in the 51 ts *gsp1* genes examined are shown by the symbol #. For comparison, the amino acid sequences of Spi1p and Ran/TC4 GTPase are aligned. The numbers indicates the amino acid positions in Gsp1p. The *boxed* 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 *Hin*dIII and then introduced into the strain N43-6C (Δ*gsp1*::*HIS3* [pRSCNR1]), in order to integrate ts *gsp1* genes into the *Hin*dIII 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 eventally 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

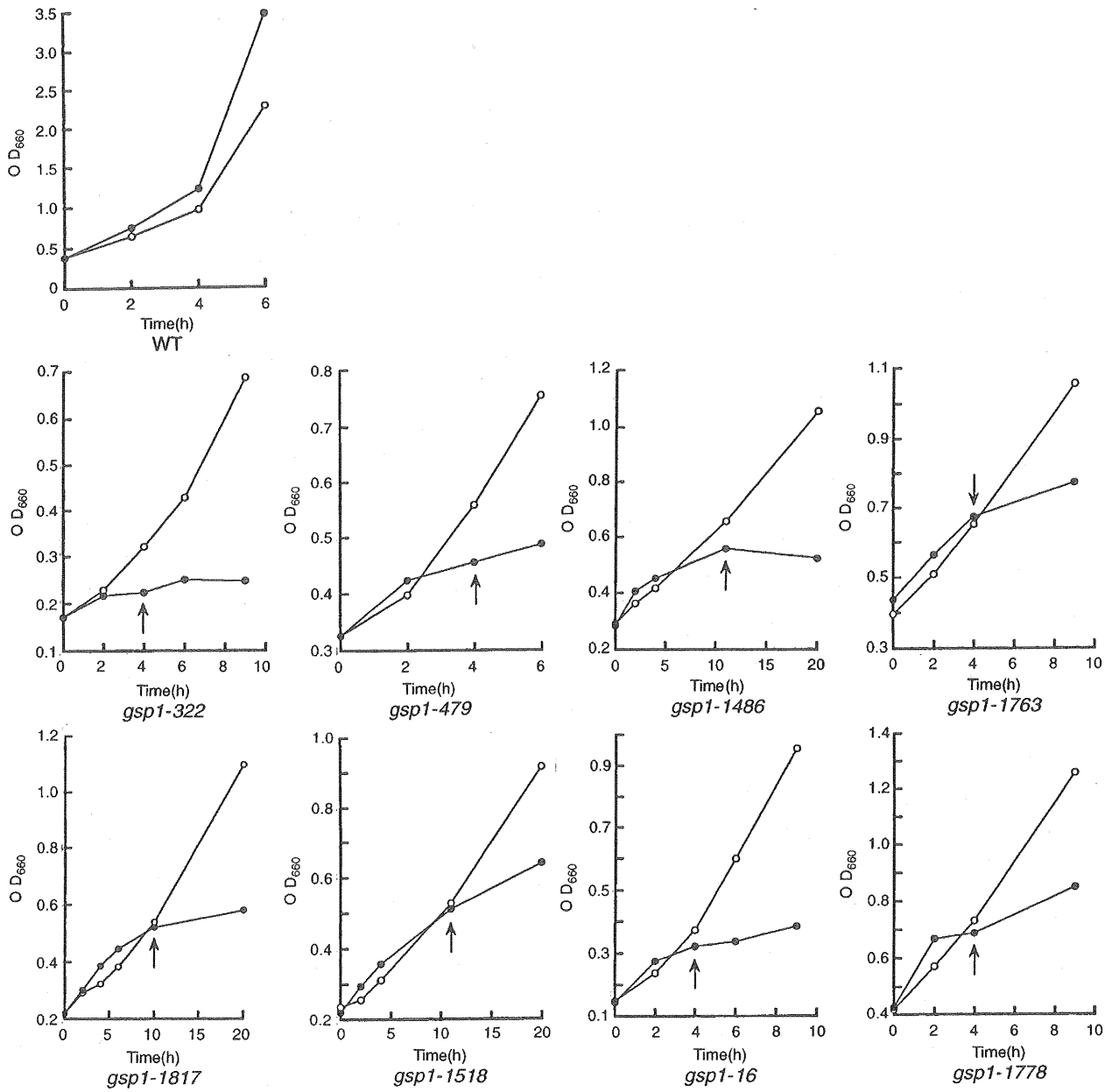


Fig. 4 Growth curves for ts *gsp1* strains. Overnight cultures of *GSP1* and *gsp1*^{ts} strains were diluted into YPD medium and grown for 4 h at 26° C. Half of each culture was then shifted to 37° C (*filled circles*) and the other half was incubated further at 26° C (*open circles*). At the indicated 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

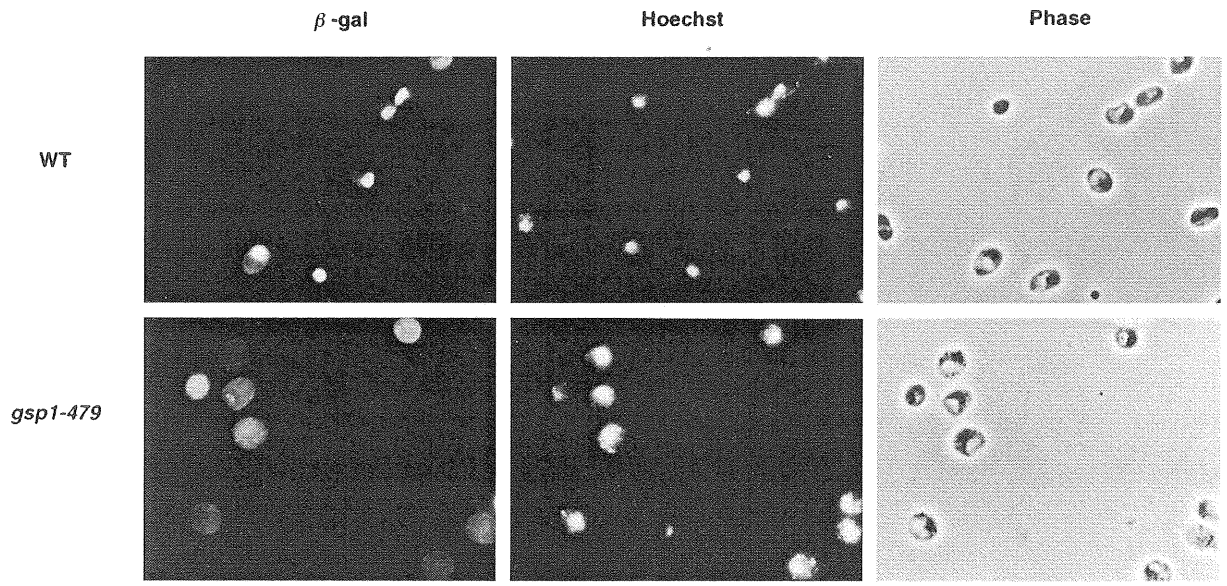


Fig. 5 Nuclear protein import in ts *gsp1* strains. Wild-type and ts *gsp1-479* strains carrying pFB1-33C were cultured in synthetic medium containing 2% raffinose, but lacking uracil, at 26° C until early log phase, and then incubated at 37° C in synthetic medium containing 2% galactose. When the growth 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 fluoresence 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.

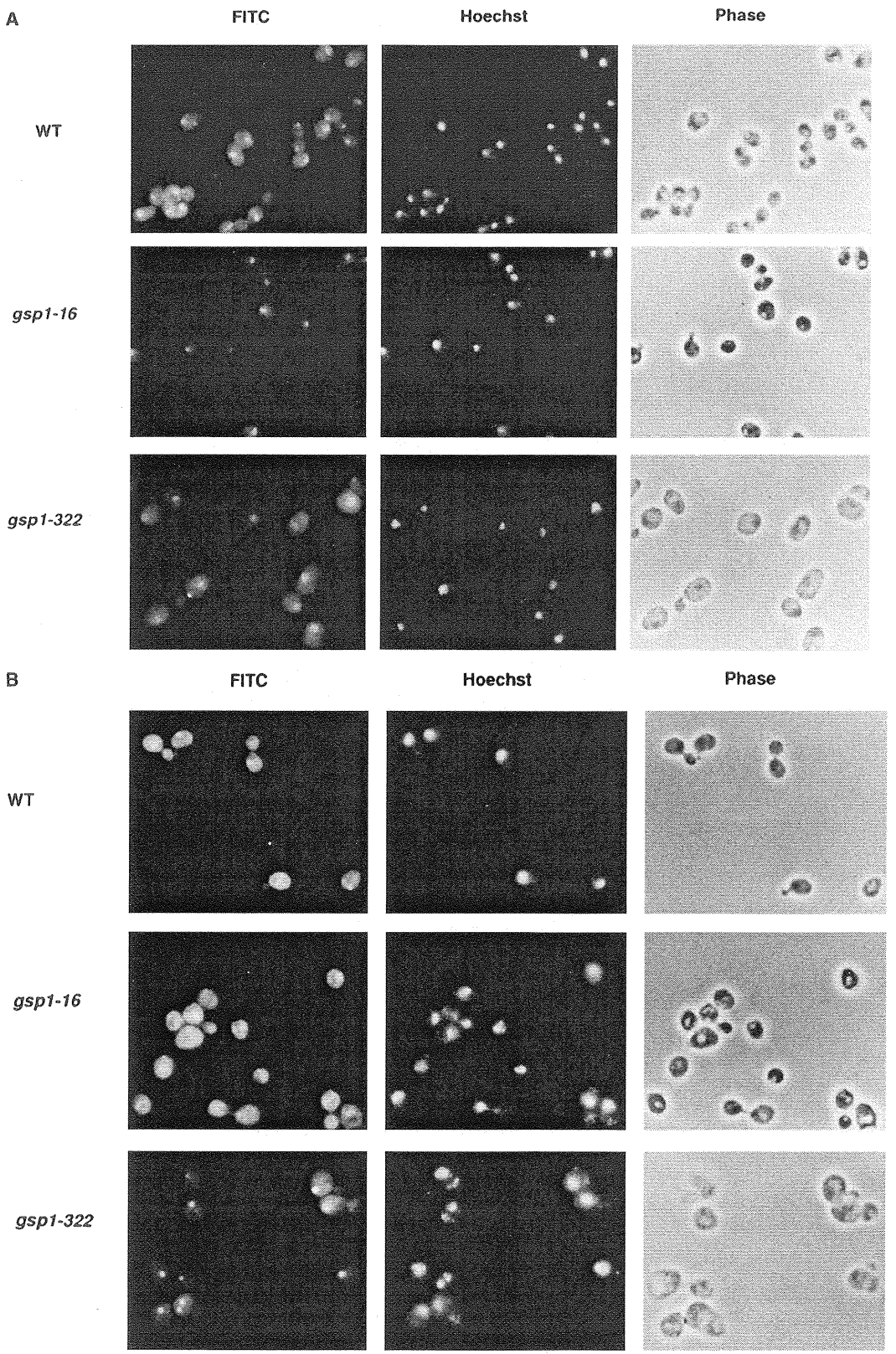
Cell cycle analysis

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


A
FITC
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WT
gsp1-16
gsp1-322
B
FITC
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Phase
WT
gsp1-16
gsp1-322

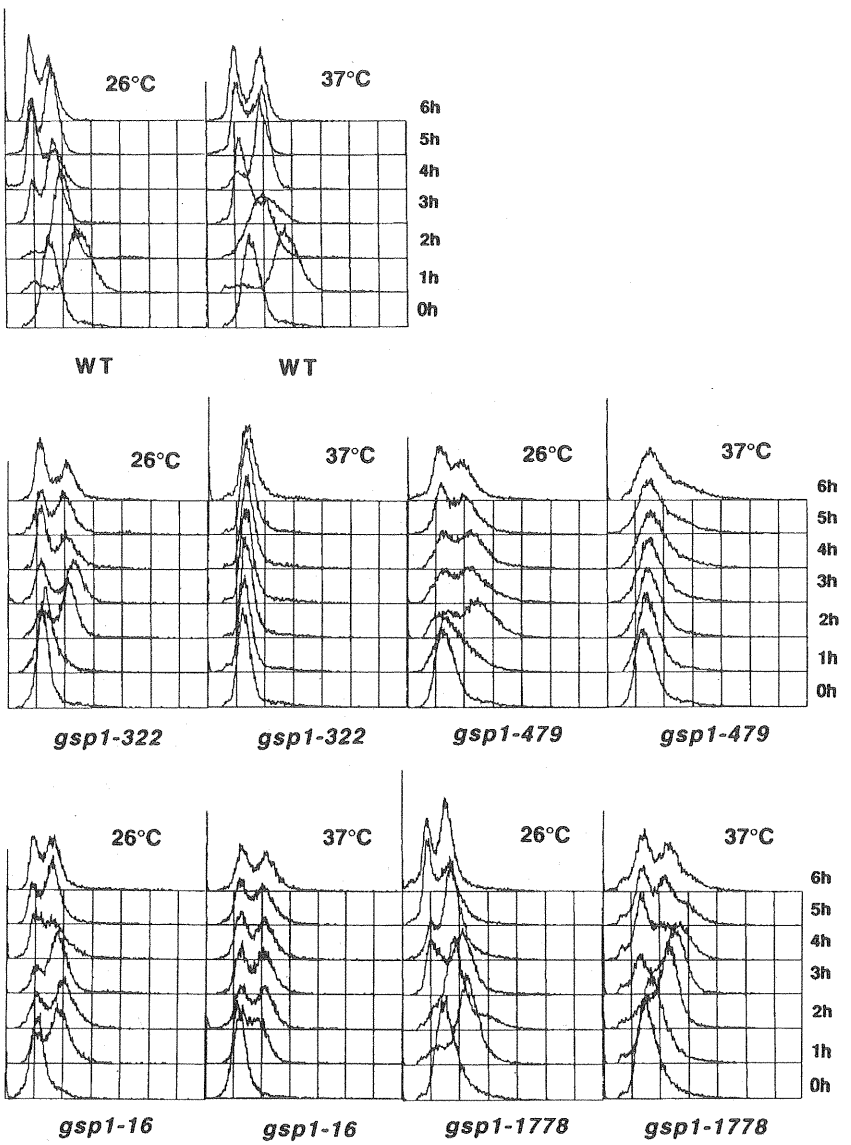

Fig. 7 Cell cycle analysis of ts *gsp1* strains. ts *gsp1* strains and wild-type strains were cultured in YPD medium at 26° C until an OD_{660nm} of 0.1 and then incubated in the presence of 10 μg/ml of α-factor for 2 h at 26° C. Subsequently, fresh α-factor (20 μg/ml final concentration) was added and 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).

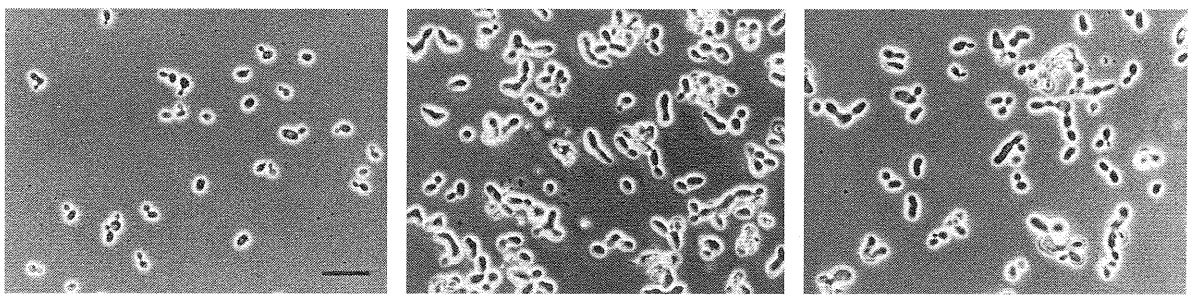

Fig. 8 Cellular morphology of ts *gsp1* cells. ts *gsp1-1757* and ts *gsp1-1778* and, as a control, wild-type strains were cultured at 26° C and photographed under phase-contrast optics. *Bar* 25 μm

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.

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