Journal of Cell Science 111, 11-21 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS3623

RagA is a functional homologue of *S. cerevisiae* Gtr1p involved in the Ran/Gsp1-GTPase pathway

Eiji Hirose, Nobutaka Nakashima, Takeshi Sekiguchi and Takeharu Nishimoto*

Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-82, Japan
*Author for correspondence (e-mail: tnishi@molbiol.med.kyushu-u.ac.jp)

Accepted 3 November 1997; published on WWW 11 December 1997

SUMMARY

Human RagA and RagB is reported to be 52% identical to a putative GTPase of *Saccharomyces cerevisiae*, Gtr1p. According to the reported nucleotide sequence, we amplified human *RagA* and *RagB*^{s} cDNAs from the human B cell cDNA library with PCR. Both cDNAs rescued a cold sensitivity of *S. cerevisiae*, *gtr1-11*. Furthermore, we introduced into the cloned human *RagA* cDNA, the mutation 'T21L' corresponding to the *gtr1-11* mutation which has been reported to suppress not only all of *rcc1*$^-$, temperature-sensitive mutants of Ran/Gsp1p GTPase GDP/GTP-exchanging factor, but also *rna1-1*, a temperature-sensitive mutant of Ran/Gsp1p GTPase-activating protein. The resulting *RagA*$^{gtr1\text{-}11}$ cDNA partially, but significantly, suppressed both *rcc1*$^-$ and *rna1-1* mutations. These results indicated that RagA and RagB^s are functional homologues of *S. cervisiae* Gtr1p. Interestingly, while wild-type human RagA and RagB^s were localized within the cytoplasm, similar to *S. cerevisiae* Gtr1p, the mutated human RagA$^{gtr1\text{-}11}$ corresponding to a dominant negative form of RagA was distributed in discrete speckles in the nucleus, being localized side by side with SC-35, a non-snRNP of the splicing complex. In contrast, a dominant positive form of RagA, Q66L was localized in the cytoplasm. Thus, RagA was suggested to shuttle between the cytoplasm and the nucleus, depending on the bound nucleotide state.

Key words: GTR1, RagA, RagB

INTRODUCTION

Mammalian Ras GTPase comprises a large family of small GTPases which function as a biological switch (Boguski and McCormick, 1993). The number of Ras-GTPase families is still increasing. Recently, by using degenerate oligonucleotides matching the PM1 and PM3 motifs of ADP ribosylation factor (ARF) as PCR primers, Schürmann et al. (1995) identified two cDNA clones, RagA and RagB, which are nearly identical and encode a novel GTPase. Both RagA and RagB, while they have no homology to the known subfamilies of mammalian Ras GTPase, have turned out to be similar to *Saccharomyces cerevisiae* Gtr1p (Bun-ya et al., 1992).

GTR1 has been isolated as a gene localized next to *Pho84* on the left arm of chromosome XIII of *S. cerevisiae*. It is not essential for growth, but its disruptant is cold-sensitive (Bun-ya et al., 1992; Nakashima et al., 1996). Gtr1p is a protein of 36 kDa which contains three motifs that are conserved in the Ras GTPase family. However, Gtr1p does not have the C-terminal lipid modification site characteristic to Ras GTPase (Boguski and McCormick, 1993), similar to Ran/Gsp1p GTPase (Drivas et al., 1990; Bischoff and Ponstingl, 1991a; Belhumeur et al., 1993). Ran was originally cloned using degenerate oligonucleotides that match the amino acid sequence conserved among Ras GTPase family members and had been designated TC4 (Drivas et al., 1990). Later on, TC4 was found to be identical to Ran (*Ra*s-like *N*uclear) GTPase which is localized within the nucleus (Bischoff and Ponstingl, 1991a).

Previously, we have isolated a cold-sensitive mutant of the *GTR1* gene, *gtr1-11* as a suppressor of *mtr1-2*, one of *S. cerevisiae rcc1*$^-$ alleles (Nakashima et al., 1996). RCC1 (*r*egulator of *c*hromosome *c*ondensation) (Ohtsubo et al., 1987) is the GDP/GTP-exchanging factor of Ran GTPase (Bischoff and Ponstingle, 1991b). Temperature-sensitive (ts) mutations of *RCC1* causes multiple phenotypes, such as splicing defect (*prp20*) (Aebi, et al., 1990), nuclear accumulation of mRNA (*mtr*) (Kadowaki et al., 1993), restoration of mating capacity to a receptorless mutant (*srm1*) (Clark and Sprague, 1989) and defects in cell cycle control (tsBN2; *pim1-d1*) (Nishimoto et al., 1978; Matsumoto and Beach, 1991; Sazer and Nurse, 1994). Overexpression of the *S. cerevisiae* Ran homologue, Gsp1p, rescues growth defect of *S. cerevisiae rcc1*$^-$ allele *prp20-1*, but neither *srm1-1* nor *mtr1-2* (Kadowaki et al., 1993; Lee et al., 1994). On the other hand, *ded1* a cold sensitive mutant of *DED1* encoding a putative ATP-dependent RNA helicase (Schmid and Linder, 1992; Chuang et al., 1997) suppresses *srm1-1* and *mtr1-2*, but not *prp20-1* (Hayashi et al., 1996). Compared to *GSP1* and *ded1*, *gtr1-11* is unique, since it suppresses not only all of *S. cerevisiae rcc1*$^-$ alleles, but also *rna1-1* a temperature-sensitive mutant of *S. cerevisiae* Gsp1p GTPase activating protein (Bischoff et al., 1995) which is

isolated as a mutant defective in RNA processing (Hutchison et al., 1969; Hopper et al., 1978). The finding that *gtr1-11* suppresses both *rcc1*$^-$ and *rna1*$^-$ suggests that Gtr1p negatively regulates the Ran/Gsp1p-GTPase cycle (Nakashima et al., 1996).

In this paper, we found that human RagA and B^s were functional homologues of *S. cerevisiae* Gtr1p. While Ran/Gsp1p GTPase is localized in the nucleus (Ren et al., 1993; Belhumeur et al., 1993), Gtr1p is localized within the cytoplasm (Nakashima et al., 1996). Similar to Gtr1p, both RagA and RagB were localized within the cytoplasm. However, the mutated form of human RagA, RagA$^{gtr1\text{-}11}$, which suppressed both *rcc1*$^-$ and *rna1*$^-$, was distributed in discrete speckles in the nucleus, similar to SC-35, a non-snRNP of the splicing complex (reviewed by Lamm and Lamond, 1993).

MATERIALS AND METHODS

Strains, plasmids and media

S. cerevisiae strains and plasmids in this study are described in Tables 1 and 2, respectively. *S. cerevisiae* was transformed by the Li-acetate method (Nakashima et al., 1996). COS-7 cells derived from the green monkey CV-1 cell line (Gluzman, 1981) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum in a humidified atmosphere containing 10% CO_2 at 37.5°C. DNA was transfected into COS-7 cells using lipofectoamine as described previously (Makishima et al., 1997).

Construction of RagA and RagB plasmids

Amplification of human RagA

Using the 5′ primer (*Sac*I-*RagA*): 5′GCAGAGCTCATGCCAA-ATACAGCCAT3′ and the 3′ primer (*RagA*-*Bam*HI): 5′GATGGA-TCCTCAACGCATAAGGAGAC3′, Human *RagA* cDNA fragments were amplified from the human B cell cDNA library (a gift from S. J. Elledge) by Pfu polymerase (Stratagene) in buffer containing 20 mM Tris-HCl, pH 8.5, 10 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 100 μg/ml BSA, 0.1% Triton X-100 and 200 μM dNTP. Amplified DNA fragments were digested with *Sac*I and *Bam*HI, and then inserted into the *Sac*I/*Bam*HI sites of pUC29 to be sequenced. *RagA* was then subcloned into the *Nco*I/*Bam*HI sites of pBluescript II TKS$^+$, resulting in HA-RagA-pBluescript II TKS$^+$. The *HA-RagA* fragment was cut out and inserted into the *Nco*I and *Bam*HI sites downstream of the *GAL1-10* promoter of pNV7, resulting in *RagA*-pNV7**.**

In order to express in mammalian cells, the *Sac*I-*Bam*HI fragment of *RagA*-pUC29 was inserted into the *Sac*I-*Bam*HI sites of pET-28a, resulting in T7-RagA-pET-28a. The *Xba*I-*Hin*dIII fragment of *T7-RagA*-pET-28a was then inserted into the *Xba*I-*Hin*dIII sites of pcDEBΔ (Nakashima et al., 1993), resulting in *T7-RagA*-pcDEBΔ.

Amplification of human RagB

From human cDNA libraries, *human RagB* cDNA was amplified with Pfu polymerase under the same conditions as that of RagA, using the 5′ primer, 5′ATGGAAGAATCTGACTC3′ and the 3′ primer, 5′AGTTTAGCGCATGAGAAGAC3′. Amplified cDNA fragments were directly inserted either into pNV7, resulting in *RagB*-pNV7, or into pET28a, resulting in *RagB*^{s}-pET28a. *RagB*^{s} of RagB^s-pET28a was then inserted into the *Xba*I and *Hin*dIII sites of pcDEBΔ, resulting in *T7-RagB*^{s}-pcDEBΔ.

Site-directed mutagenesis

RagA was amplified using the 5′ primer 5′CAATTCCCCTCTAG-AAATAATTTTG3′ and the 3′ primer 5′CCCGATATCTCAACGCAT-AAGGAGAC3′ in Ampligase buffer (20 mM Tris-HCl, pH 8.3, 25 mM KCl, 15 mM $MgCl_2$, 1.5 mM NAD and 0.01% Triton-X-100) containing the mutagenic primer p-AGAGCGGGTCGGGGAAGC-TTAGCATGAGGTCGATAAT, dNTP, Ampligase (Epicentre) and Taq DNA polymerase, by PCR. Amplified DNA was digested with *Xba*I and *Eco*RV, and then inserted into the *Xba*I/*Eco*RV site of pcDEBΔ.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
NN7-3B	*MATα gtr1-11 ade2 ura3 leu2*	This study
N51-10A	*MAT***a** *prp20-1 ura3 his3 leu2 trp1*	Hayashi et al. (1996)
SY1115	*MATα srm1-1 ura3 his4 leu2-3,112 trp1*	Clark and Sprague (1989)
N48-5D	*MATα mtr1-2 ade2 ura3 leu2 trp1*	This study
NN19-5B	*MAT***a** *rna1-1 ade2 ura3 leu2 his3 trp1*	Noguchi et al. (1997)
NOY612	*MAT***a** *srp1-31 ade2-1 ura3-1 his3-11 leu2-3,112 trp1-1 can1-100*	Yano et al. (1994)
NOY388	*MAT***a** *ade2-1 ura3-1 his3-11 leu2-3,112 trp1-1 can1-100*	Yano et al. (1994)

Table 2. Yeast and mammalian plasmids used in this study

Plasmid	Marker	Comment	Source
pL48	2μ *URA3 GAL1-10:HA-GTR1*	*Xho*I/*Spe*I fragment from pL46 cloned into pNV7*	This study
RagA-pNV7	2μ *URA3 GAL1-10:HA-RagA*	*HA*-tag fused to *RagA* in frame cloned into pNV7*	This study
RagB^s-pNV7	2μ *URA3 GAL1-10:RagB*^{s}	*Xho*I/*Spe*I-treated PCR fragment cloned into pNV7*	This study
RagB^n-pNV7	2μ *URA3 GAL1-10:RagB*^{n}	*Xho*I/*Spe*I-treated PCR fragment cloned into pNV7*	This study
T7-RagA-pNV7	2μ *URA3 GAL1-10:T7-RagA*	*Xba*I fragment from *RagA*-pET-28a cloned into pNV7*	This study
T7-RagB^s-pNV7	2μ *URA3 GAL1-10:T7-RagB*^{s}	*Xba*I/*Eco*RV fragment from *T7-RagB*^{s}-pcDEBΔ cloned into pNV7*	This study
T7-RagB^n-pNV7	2μ *URA3 GAL1-10:T7-RagB*^{n}	*Xba*I/*Eco*RV fragment from *T7-RagB*^{n}-pcDEBΔ cloned into pNV7*	This study
RagA$^{gtr1\text{-}11}$-pGAP	2μ *TRP1 GAP:RagA*$^{gtr1\text{-}11}$	*Bam*HI/*Eco*RV fragment from *RagA*$^{gtr1\text{-}11}$-pcDEBΔ cloned into pGAP†	This study
RagA$^{gtr1\text{-}11}$-pGAP314	*CEN6 TRP1 GAP:RagA*$^{gtr1\text{-}11}$	*Eco*RI/*Eco*RV fragment from *RagA*$^{gtr1\text{-}11}$-pcDEBΔ cloned into pGAP314‡	This study
T7-RagA-pcDEBΔ	HB^r SRα*:T7–RagA*	*Xba*I/*Hin*dIII fragment from *RagA*-pET-28a cloned into pcDEBΔ§	This study
T7-RagB^s-pcDEBΔ	HB^r SRα*:T7–RagB*^{s}	*Xba*I/*Hin*dIII fragment from *RagB*^{s}-pET-28a cloned into pcDEBΔ§	This study
T7-RagB^n-pcDEBΔ	HB^r SRα*:T7–RagB*^{n}	*Xba*I/*Hin*dIII fragment from *RagB*^{n}-pET-28a cloned into pcDEBΔ§	This study
T7-RagA$^{gtr1\text{-}11}$-pcDEBΔ	HB^r SRα*:T7–RagA*$^{gtr1\text{-}11}$	*T7–RagA*$^{gtr1\text{-}11}$ cloned into pcDEBΔ§	This study
FLAG-RagA$^{gtr1\text{-}11}$-pcDEBΔ	HB^r SRα*:FLAG–RagA*$^{gtr1\text{-}11}$	*FLAG*-tag fused to *RagA*$^{gtr1\text{-}11}$ (see Materials and Methods)	This study
T7-RagA^{Q66L}-pcDEBΔ	HB^r SRα*:T7–RagA*^{Q66L}	*T7–RagA*^{Q66L} cloned into pcDEBΔ§	This study

*pNV7: yeast expression vector containing *GAL1-10* promotor (Ninomiya-Tsuji et al., 1991).
†pGAP: yeast expression vector containing *TDH3* promotor (Tanaka et al., 1988).
‡pGAP314: yeast expression vector containing *TDH3* promotor (Noguchi et al., 1997).
§pcDEBΔ: mammalian expression vector containing SRα promotor (Nakashima et al., 1993).

Construction of FLAG -RagA$^{gtr1\text{-}11}$-pcDEBΔ

*Xba*I-FLAG (plus): 5′CCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGACTACAAGGACGACGATGACAAGGGTGGCGGTGGCGGTGAGCTCTAG3′, and *Xba*I-FLAG (minus): 5′CTAGAGCTCACCGCCACCGCCACCCTTGTCATCGTCGTCCTTGTAGTCCATGGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGG3′ (prepared by Greiner Japan) were annealed, digested with *Sac*I and then ligated to *RagA$^{gtr1\text{-}11}$*-pcDEBΔ which had been digested with *Sac*I. The obtained FLAG-tagged RagA$^{gtr1\text{-}11}$ contains the amino acid sequence of the FLAG tag, MDYDDDDK (Hopp et al., 1988), and of glycine-linker (Guan and Dixon, 1991) at the N terminus.

Construction of T7-RagA^{Q66L}-pcDEBΔ

The *Bgl*II-*Bam*HI fragment of *T7-RagA*-pcDEBΔ was subcloned into the *Bam*HI site of pkF18k and amplified using GACTGTGGCGGTCTGGACACCTTCATG as the mutagenic primer and the Site-directed Mutagenesis System (Takara). The *Xba*I-*Eco*RV fagment of pkF18k containing the resulting *T7-fused RagA^{Q66L}* was then inserted into pCDEBΔ.

Indirect immunochemistry

Cells were fixed with cold methanol (−20°C), permeabilized with 1% Tween-20 and incubated in PBS buffer containing 3% BSA (fraction five; Sigma), 0.5% gelatin (Telestean gelatin; Sigma) and 0.2% Tween-20. Cells were then exposed to the primary antibody, T7 tag monoclonal antibody (mAb) (Novagen) and then treated with the secondary antibody, FITC-conjugated goat anti-mouse IgG antibody. When doubly stained, fixed cells were treated first with the mouse mAb to SC-35 (Pharmingen) and stained with the secondary antibody conjugated to fluorochrome. After incubation in blocking PBS buffer, fixed cells were secondarily treated with the anti-FLAG rabbit-polyclonal antibodies (Santa Cruz) and stained with the secondary antibody conjugated to fluorochrome. The secondary antibodies used were Texas red-conjugated sheep anti-mouse IgG antibody (Amersham), FITC-conjugated goat anti-mouse IgG antibody (Tago), FITC-conjugated goat anti-rabbit IgG antibody (Tago) and Texas red-conjugated goat anti-rabbit IgG antibody (Amersham). Cells were finally stained with Hoechst 33342 and then mounted on Vectashield (Vector).

Microscopy and image analysis

Zeiss Axio Photo was used for sample analysis by the standard microscopic method. Digital imaging of stained cells was obtained using the Olympus laser-scanning microscope LSM-GB200 system. The X-Y scanning was performed and directly transferred to color reversal films using the film recorder (Avio) FR-3000-SP. Optical sections of nuclei were obtained in 0.5-micron steps using the X-Y-Z scanning mode of LSM-GB200 and printed by pictrography 3000 (Fujix) through Adobe Photoshop^{TM} 3.0J.

RESULTS

Amplification of RagA and RagB

According to the nucleotide sequence of human *RagA* and *RagB* (GenBank accession numbers X90529 and X90530, respectively) (Schürmann et al., 1995), the 5′ and 3′ primers for PCR were constructed. Using these primers, cDNA fragments were amplified from the human B cell cDNA library. After sequencing, these cDNA fragments were found to contain some differences between the obtained and the deposited nucleotide sequences of human *RagA* and *RagB*, as follows.

Amplified *RagA* cDNA encoded a protein identical to the human RagA sequence, except that the third nucleotide of codon 181, cytosine, was found to be thymine in the newly amplified *RagA* cDNAs, while the amino acid was not changed. On the other hand, amplified *RagB^{s}* cDNA encoded a protein which differed from the human RagB reported (Schürmann et al., 1995) by a single amino acid, valine (codon 309), which was changed to methione in the obtained human RagB^{s}. In addition, the third nucleotide in codon 228, adenine, was changed to thymine in the amplified cDNA. The nucleotide changes mentioned above were found in all of more than 6 independently isolated cDNA fragments.

The alternative splicing form of RagB^{l} which has been reported by Schürmann et al. (1995) was not amplified. Instead, we obtained another form of RagB, designated *RagB^{n}*, which possessed an extra nucleotide sequence of 108 base pairs between codons 204 and 205 of human RagB^{s} (Fig. 1A). Due to a termination codon within the inserted sequence, the C-terminal tail of *RagB^{n}* was truncated, similar to that of *gtr1-3*Δ (Bun-ya et al., 1992).

Amplified human RagA and RagB cDNA fragments were engineered to fuse in frame with the T7-tag at the N terminus, and then expressed in COS cells. Immunoblotting analysis using the monoclonal antibody (mAb) to the T7 tag revealed that these cDNAs expressed proteins which possessed molecular masses of 40.6 kDa (RagA), 33.2 kDa (RagB^{n}) and 44.3 kDa (RagB^{s}), estimates based on the nucleotide sequence (Fig. 1B).

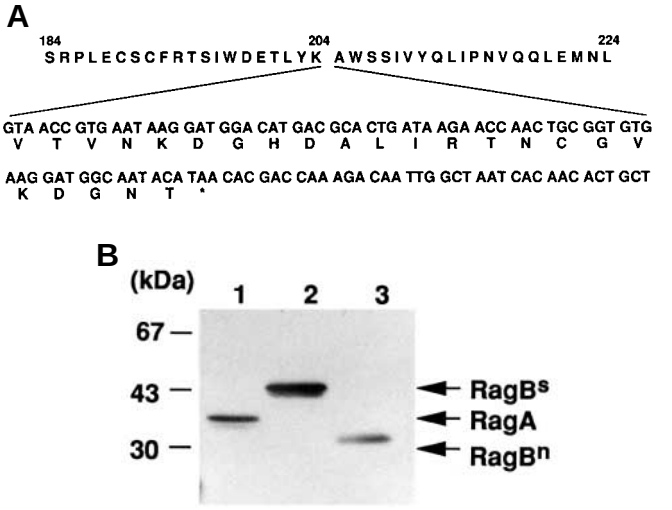

Fig. 1. Amplified human RagA and RagB. (A) Structure of RagB^{n}: the nucleotide and amino acid sequences inserted into RagB^{s} are shown. (B) Expression of amplified Rag cDNAs in COS cells. COS cells were seeded at a density of 7×10^{5}/50 mm dish. The next day, 8 μg of DNA (T7-RagA (lane 1), T7-RagB^{s} (lane 2) and T7-RagB^{n} (lane 3) carried on pcDEBΔ) were transfected into COS cells as described in Materials and Methods. After incubation at 37°C for 48 hours, transfected cells were harvested and the crude lysates were prepared. Total cellular proteins were separated by SDS-12.5% polyacrylamide gel electrophoresis and analysed by immunoblotting using the mAb to T7-tag. Arrows indicate the positions of expressed proteins.

Both *RagA* and *RagB^{s}* rescued a growth defect of *S. cerevisiae gtr1-11*

In order to address the question of whether human RagA and

RagB are functional homologues of *S. cerevisiae* Gtr1p, the obtained human *RagA* and *RagB* cDNAs were inserted into a site downstream of the *GAL1-10* promoter of *S. cerevisiae* expression vector, pNV7. The resulting *RagA*- and *RagB*-pNV7 were introduced into the *S. cerevisiae gtr1-11* strain, NN7-3B by the Li-acetate method. Transformants were selected in synthetic medium containing 2% dextrose, but lacking uracil. Isolated Ura^+ transformants grown in the same synthetic medium were plated onto synthetic medium containing 2% galactose, but lacking uracil, at 14°C, the nonpermissive temperature and at 30°C, the permissive temperature for NN7-3B cells (Nakashima et al., 1996), respectively.

NN7-3B cells expressing either RagA or $RagB^s$ grew well at 14°C the nonpermissive temperature as well as at 30°C, the permissive temperature (Fig. 2A and B). However, $RagB^n$ cDNA encoding a protein lacking the C-terminal tail of $RagB^s$ did not seem to rescue the cold sensitivity of *gtr1-11* (Fig. 2B). In order to confirm the effect of $RagB^s$ and $RagB^n$ on *gtr1-11*, equal volumes of serially diluted cultures of Ura^+ transformants were dropped on plates, and cells were incubated at 14°C. While several colonies papillated from transformants containing either pL48(*GTR1*) or $RagB^s$-pNV7($RagB^s$) after a 1:16 dilution, no NN7-3B cells possessing the $RagB^n$-pNV7($RagB^n$) or pNV7 vector alone (Vector) grew after a 1:4 dilution (data not shown). Thus, *RagA* and $RagB^s$ cDNAs complemented the growth defect of *gtr1-11*, whereas $RagB^n$ did not.

RagA and $RagB^s$ are localized within the cytoplasm

S. cerevisiae Gtr1p is localized within the cytoplasm (Nakashima et al., 1996). In order to examine the localization of RagA and $RagB^s$, both T7-tagged *RagA* and $RagB^s$ cDNAs, which rescued the cold sensitivity of *gtr1-11* were inserted into the mammalian expression vector pcDEBΔ and then transfected into COS cells. Expression of transfected human RagA and $RagB^s$ was detected from 24 hours after transfection by immunoblotting analysis. We fixed transfected cells at 48 hours after transfection and stained with the mAb to T7-tag.

In COS cells transfected either with *RagA* or $RagB^s$ cDNA, the antibody to T7-tag gave rise to punctate staining throughout the cytoplasm (Fig. 3). In the same visual field, cells which did not express Rag proteins were not stained with the mAb to the T7 tag, indicating that the observed staining is specific to T7-tagged proteins. Taking these results together, RagA and $RagB^s$ were concluded to be localized in the cytoplasm.

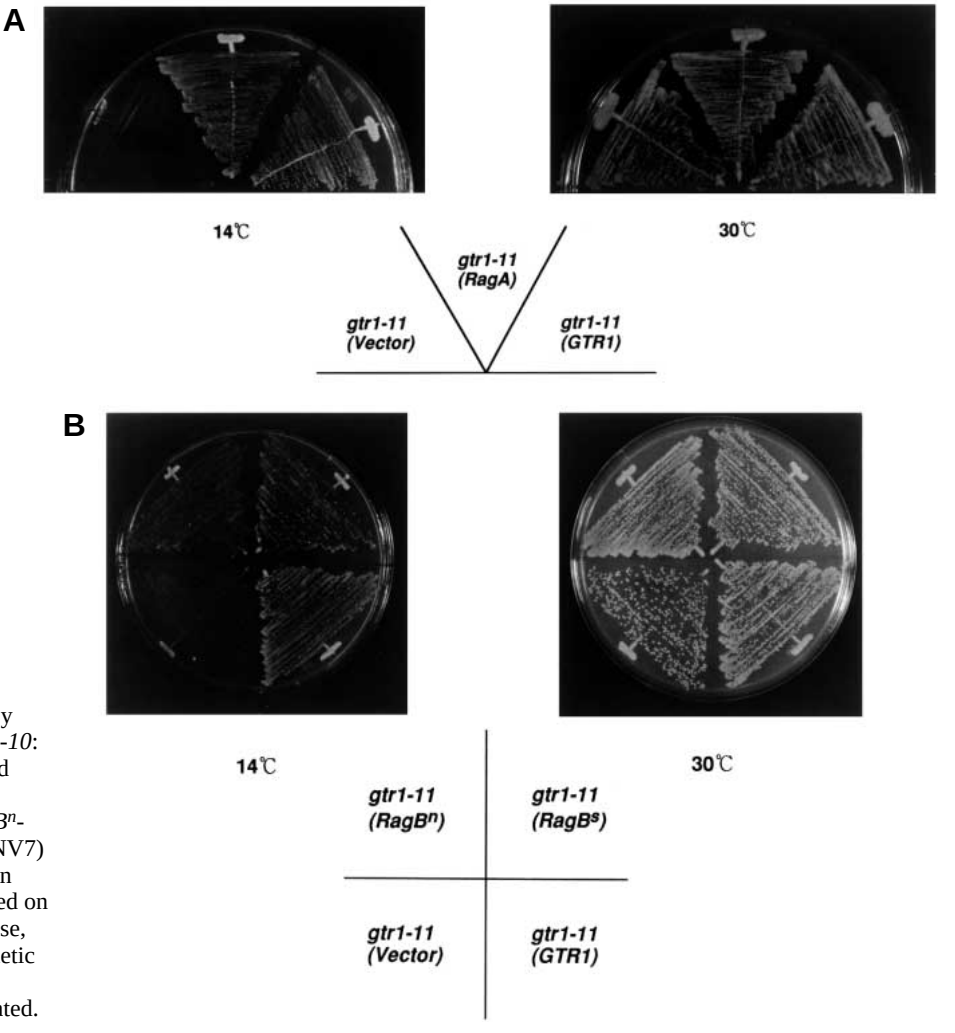

Fig. 2. Complementation analysis of *gtr1-11* by human RagA, $RagB^s$ and $RagB^n$. pL48 (*GAL1-10*: *GTR1*), *HA-RagA*-pNV7 (*GAL1-10*: *RagA*) and vector alone (pNV7) (A), or pL48 (*GAL1-10*: *GTR1*), $RagB^s$-pNV7 (*GAL1-10*: $RagB^s$), $RagB^n$-pNV7 (*GAL1-10*: $RagB^n$) and vector alone (pNV7) (B) were introduced into the *S. cerevisiae* strain NN7-3B (*gtr1-11*). Transformants were selected on synthetic medium plates containing 2% dextrose, but lacking uracil, and were re-plated on synthetic medium plates containing 2% galactose, but lacking uracil at either 14°C, or 30°C as indicated.

Human *Rag$^{gtr1\text{-}11}$* mutant partially rescued *S. cerevisiae rcc1^{-}* and *rna1-1*

The cold-sensitive mutant of *S. cerevisiae*, *gtr1-11*, was originally isolated by Nakashima et al. (1996), as a suppressor of *S. cerevisiae mtr1-2*, one of the *rcc1*$^{-}$ alleles (Kadowaki et al., 1993). Later on, *gtr1-11* has been found to suppress not only all of the *rcc1*$^{-}$ alleles, but also *rna1-1* (Nakashima et al., 1996). If mammalian RagA and RagB^{s} are real functional homologues of *S. cerevisiae* Gtr1p, RagA and RagB^{s} possessing the mutation corresponding to *gtr1-11* could rescue the temperature-sensitive growth defects of *S. cerevisiae rcc1*$^{-}$ and *rna1*$^{-}$. In order to address this issue, the *gtr1-11* mutation, S20L (N. Nakashima et al., unpublished) was introduced into the corresponding Thr^{21} site of human *RagA* cDNA by site-directed mutagenesis using Ampligase (Michael, 1994). The amino acid sequence around the *gtr1-11* mutation is identical between Gtr1p and RagA except for the amino acid residue of mutation site which is chemically conserved (Fig. 4A). The introduction of the 'T21L' mutation into human RagA cDNA was confirmed by the creation of a *Hin*dIII site (Fig. 4A, *AAGCTT*) in the *RagA* open reading frame. The mutated human *RagA* cDNA, designated *RagA$^{gtr1\text{-}11}$*, was inserted into the single- and multi-copy *S. cerevisiae* expression vectors, pGAP314 (Noguchi et al., 1997) and pGAP, respectively. The resulting plasmids were introduced into *S. cerevisiae rcc1*$^{-}$ strains N51-10A (*prp20-1*, *trp1*), SY115 (*srm1-1*, *trp1*) and N48-5D (*mtr1-2*, *trp1*), and the *rna1*$^{-}$ strain, NN19-5B (*rna1-1*, *trp1*). Transformants were selected in synthetic medium containing 2% dextrose, but lacking tryptophan.

Trp^{+} transformants of *rcc1*$^{-}$ strains were plated onto the same synthetic medium either at 30.5-31°C, the nonpermissive temperature or at 23-26°C, the permissive temperature, as indicated in Fig. 4B. *RagA$^{gtr1\text{-}11}$* carried on the multicopy vector pGAP rescued the temperature-sensitive growth defects of all of the *S. cerevisiae rcc1*$^{-}$ alleles examined, whereas the vector alone did not. The suppression of *rcc1*$^{-}$ by *RagA$^{gtr1\text{-}11}$* is dependent upon the expressed dose of RagA$^{gtr1\text{-}11}$, since *RagA$^{gtr1\text{-}11}$* carried on a single-copy plasmid did not rescue the growth defect of any *rcc1*$^{-}$ alleles (data not shown). Furthermore, overexpression of RagA$^{gtr1\text{-}11}$ rescued the temperature sensitive growth defect of *rna1-1* at 28°C (Fig. 4C). Taking the above results together, *RagA$^{gtr1\text{-}11}$* partly but significantly rescued the temperature-sensitive growth defects of both *rcc1*$^{-}$ and *rna1*$^{-}$ strains.

The next question is whether overexpression of RagA$^{gtr1\text{-}11}$ can rescue defects in the nuclear pore transport function, since both *rcc1*$^{-}$ and *rna1*$^{-}$ have defects in the nuclear/cytoplasmic transport of macromolecules (reviewed by Sazer, 1996; Seki et al., 1996). In order to address this issue, *RagA$^{gtr1\text{-}11}$* cDNA was introduced into the *S. cerevisiae srp1-31* strain NOY612, a ts mutant of the *S. cerevisiae* importin α homologue (Yano et al., 1994) and as a control, into the parental strain NOY388. While parental strains carrying *RagA$^{gtr1\text{-}11}$*-pGAP grew well either at 23°C, the permissive temperature, or at 30°C, the nonpermissive temperature, *srp1-31* carring *RagA$^{gtr1\text{-}11}$*-pGAP did not grow at 30°C (Fig. 4D). Thus, *RagA$^{gtr1\text{-}11}$* cDNA could not rescue a growth-defect of *srp1-31* strain.

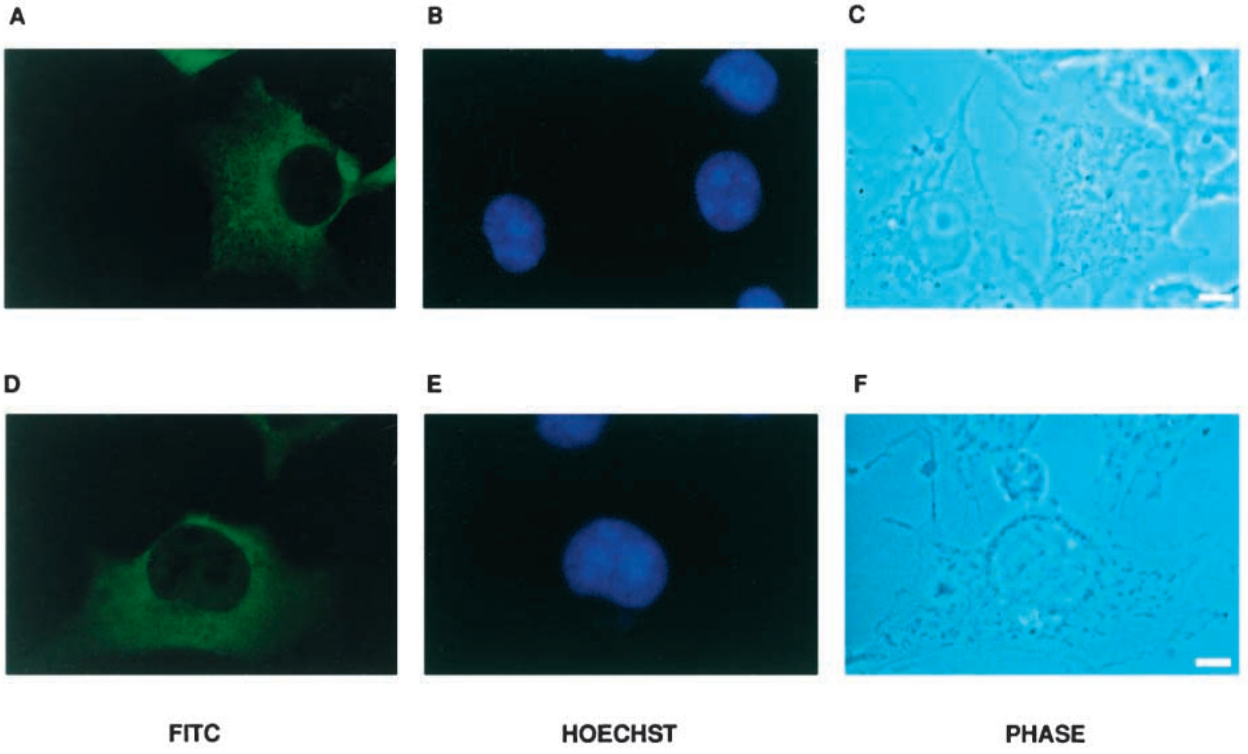

Fig. 3. Localization of human RagA and RagB^{s} in COS cells. *T7-RagA* (A-C) and *T7-RagB^{s}* (D-F)-pcDEBΔ DNAs were transfected into COS cells. After incubation at 37°C for 48 hours, transfected cells were fixed with cold methanol (–20°C), stained first with the mAb to T7 tag, and then with FITC-conjugated goat anti-mouse IgG antibody as described in Materials and Methods. Finally cells were stained with Hoechst 33342. Cells within the same visual field are demonstrated by T7-tag staining (FITC), Hoechst and phase-contrast. Bar, 20 μm.

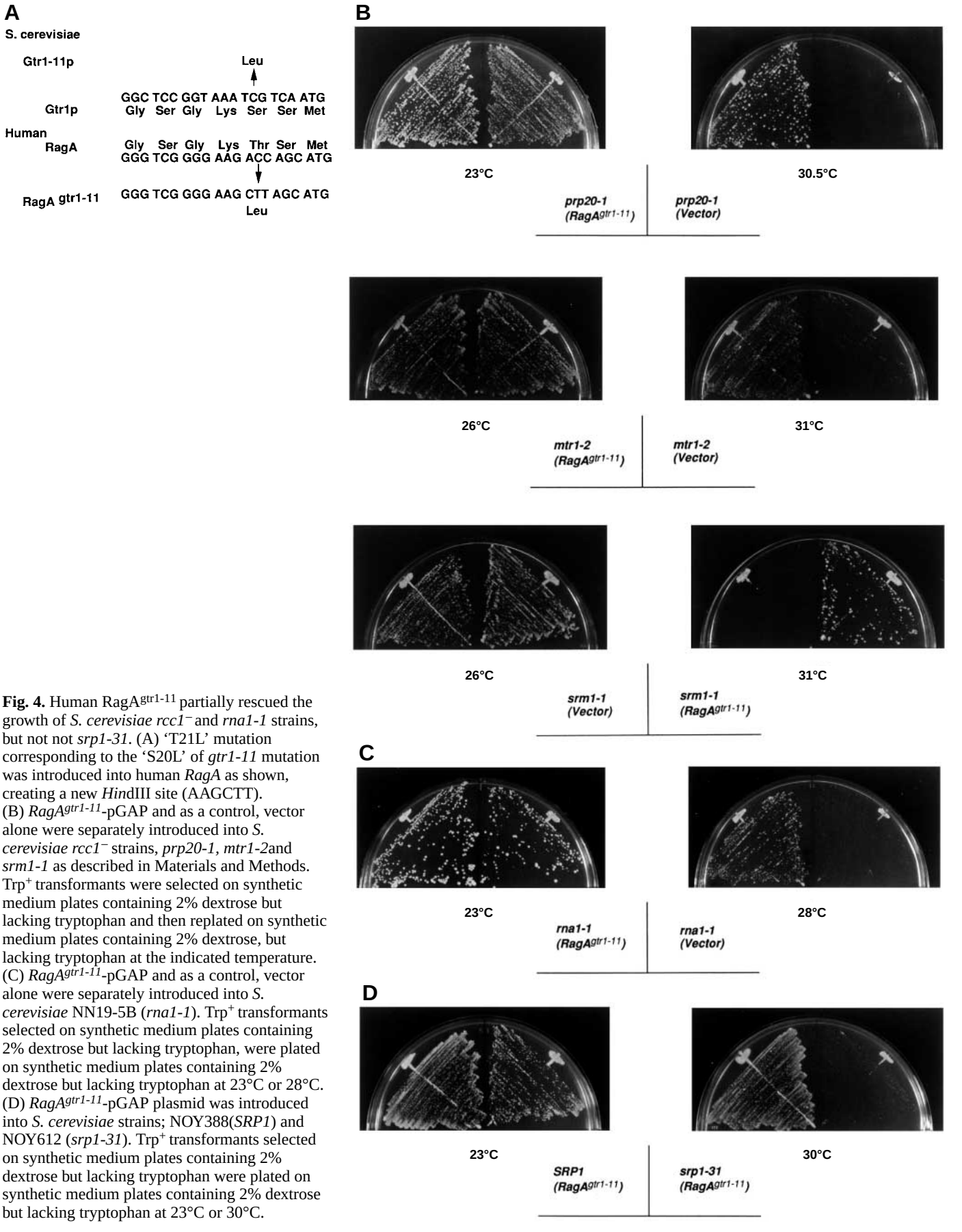

Fig. 4. Human RagA$^{gtr1\text{-}11}$ partially rescued the growth of *S. cerevisiae rcc1*$^{-}$ and *rna1-1* strains, but not not *srp1-31*. (A) 'T21L' mutation corresponding to the 'S20L' of *gtr1-11* mutation was introduced into human *RagA* as shown, creating a new *Hin*dIII site (AAGCTT). (B) *RagA*$^{gtr1\text{-}11}$-pGAP and as a control, vector alone were separately introduced into *S. cerevisiae rcc1*$^{-}$ strains, *prp20-1, mtr1-2*and *srm1-1* as described in Materials and Methods. Trp^{+} transformants were selected on synthetic medium plates containing 2% dextrose but lacking tryptophan and then replated on synthetic medium plates containing 2% dextrose, but lacking tryptophan at the indicated temperature. (C) *RagA*$^{gtr1\text{-}11}$-pGAP and as a control, vector alone were separately introduced into *S. cerevisiae* NN19-5B (*rna1-1*). Trp^{+} transformants selected on synthetic medium plates containing 2% dextrose but lacking tryptophan, were plated on synthetic medium plates containing 2% dextrose but lacking tryptophan at 23°C or 28°C. (D) *RagA*$^{gtr1\text{-}11}$-pGAP plasmid was introduced into *S. cerevisiae* strains; NOY388(*SRP1*) and NOY612 (*srp1-31*). Trp^{+} transformants selected on synthetic medium plates containing 2% dextrose but lacking tryptophan were plated on synthetic medium plates containing 2% dextrose but lacking tryptophan at 23°C or 30°C.

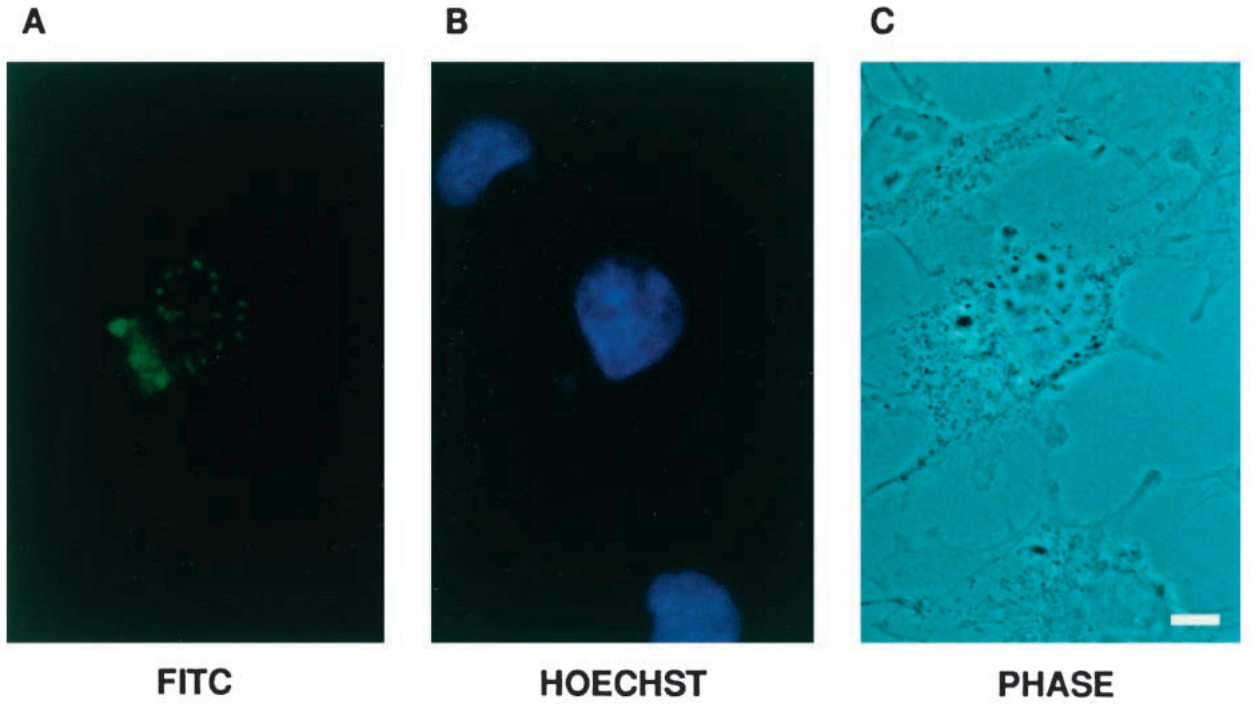

Fig. 5. Localization of human RagA$^{gtr1\text{-}11}$ in COS cells. *T7-RagA$^{gtr1\text{-}11}$*-pcDEBΔ was transfected into COS cells. After incubation at 37°C for 48 hours, transfected cells were fixed with cold methanol (–20°C), treated with T7-tag mAb and then with FITC-conjugated goat anti-mouse IgG antibody as described in Materials and Methods. Finally cells were stained with Hoechst 33342. Cells in the same visual field are demonstrated by T7-tag staining (FITC), Hoechst and phase-contrast. Bar, 20 μm.

Mutated human RagA accumulated adjacent to SC35 in the nucleus

In order to clarify the mechanism by which *gtr1-11* suppresses *rcc1*$^-$ and *rna1*$^-$, we investigated the localization of RagA$^{gtr1\text{-}11}$. To do that, T7-tag was fused in frame at the N terminus of human RagA$^{gtr1\text{-}11}$ cDNA. The resultant T7-*RagA$^{gtr1\text{-}11}$* cDNA was inserted into the mammalian expression vector, pcDEBΔ and transfected into COS cells. Transfected *RagA$^{gtr1\text{-}11}$* was expressed in COS cells with a kinetics similar to that of wild-type *T7-RagA*. Expression of *RagA$^{gtr1\text{-}11}$* was detected at 24 hours after transfection and became a plateau after 48 hours, by immunoblotting analysis (data not shown). At 48 hours after transfection, cells were fixed and these fixed cells were treated with the mAb to T7-tag. In contrast to wild-type RagA which was localized in the cytoplasm, mutated human RagA$^{gtr1\text{-}11}$ proteins were localized in the nucleus (Fig. 5). Interestingly, in the nucleus, RagA$^{gtr1\text{-}11}$ proteins were distributed in speckles similar to those of splicing complexes (Xing et al., 1993; Misteli and Spector, 1997).

In order to investigate the relationship between RagA$^{gtr1\text{-}11}$ and the splicing complex, the FLAG-tag instead of the T7-tag, was fused in frame to the N terminus of *RagA$^{gtr1\text{-}11}$* cDNA. In this way, we could doubly stain cells with the mouse monoclonal antibody against a marker of splicing complexes and with the rabbit antibody to the FLAG-tag. The resultant *FLAG-RagA$^{gtr1\text{-}11}$* cDNA was inserted into a mammalian expression vector pcDEBΔ and then introduced into COS cells. Transfected COS cells were fixed after incubation for 48 hours and the fixed cells were doubly stained with the anti-FLAG tagged antibody and the mAb to SC-35, a non-snRNP of the splicing complex (Fu and Maniatis, 1990). Three representative figures are shown in Fig. 6A. In the top panel, red and green fluorescence were used to stain RagA$^{gtr1\text{-}11}$ and SC-35, respectively. In the middle and bottom panels, green and red fluorescence were used to stain RagA$^{gtr1\text{-}11}$ and SC-35, respectively. In the nucleus, FLAG-tagged RagA$^{gtr1\text{-}11}$ and SC-35 were distributed non-homogeneously in speckles. When superimposed, speckles of RagA$^{gtr1\text{-}11}$ were found to be co-located (top and bottom panels) and overlapping (middle panel), with those of SC-35.

In order to further confirm the spatial relationship between RagA$^{gtr1\text{-}11}$ and SC-35, the area squared in Fig. 6B was optically cut at 1.0-micron intervals in the Z-axis using digital imaging microscopy and was then aligned from the bottom to the top along the Z-axis (Fig. 6C). Except for a few areas, RagA$^{gtr1\text{-}11}$ does not occupy the same area as SC-35. As shown in both a and b of Fig. 6C, SC-35 and RagA$^{gtr1\text{-}11}$ were localized side by side on the same optical sections. SC-35 was not co-immunoprecipitated with RagA$^{gtr1\text{-}11}$ by the antibody to T7 tag, and RagA$^{gtr1\text{-}11}$ was not immunoprecipitated by the antibody to SC-35 (data not shown). Thus, there seemed to be no direct interaction between SC-35 and RagA$^{gtr1\text{-}11}$.

Cellular localization of RagA changed depending on the nucleotide bound form

The *gtr1-11* mutation, S20L corresponds to a dominant negative form of Ran, T24N (N. Nakashima et al., unpublished). The difference in cellular localization between the wild-type RagA and the mutant RagA$^{gtr1\text{-}11}$, thus, may depend on the bound nucleotide state of RagA. In order to address this issue, we constructed the Q66L mutant of RagA corresponding to a dominant positive form of Ran Q69L. Three different forms of

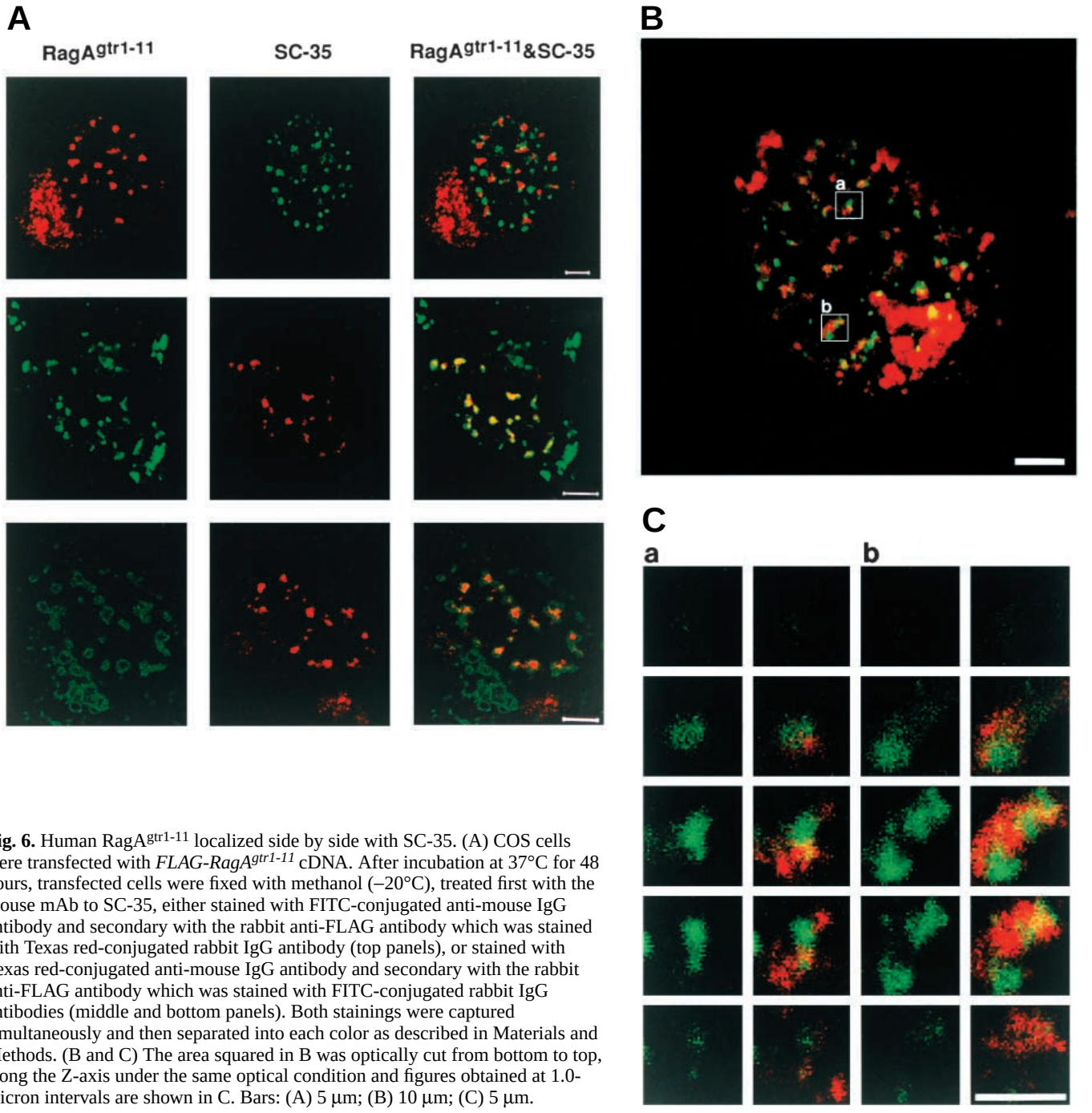

Fig. 6. Human RagA$^{gtr1-11}$ localized side by side with SC-35. (A) COS cells were transfected with *FLAG-RagA$^{gtr1-11}$* cDNA. After incubation at 37°C for 48 hours, transfected cells were fixed with methanol (–20°C), treated first with the mouse mAb to SC-35, either stained with FITC-conjugated anti-mouse IgG antibody and secondary with the rabbit anti-FLAG antibody which was stained with Texas red-conjugated rabbit IgG antibody (top panels), or stained with Texas red-conjugated anti-mouse IgG antibody and secondary with the rabbit anti-FLAG antibody which was stained with FITC-conjugated rabbit IgG antibodies (middle and bottom panels). Both stainings were captured simultaneously and then separated into each color as described in Materials and Methods. (B and C) The area squared in B was optically cut from bottom to top, along the Z-axis under the same optical condition and figures obtained at 1.0-micron intervals are shown in C. Bars: (A) 5 μm; (B) 10 μm; (C) 5 μm.

T7-fused RagA, wild-type RagA, RagA$^{gtr1-11}$ and RagA^{Q66L}, were introduced into hamster BHK21 cells. Transfected cells were stained with the anti-T7 antibody. As shown in Fig. 7, both wild-type RagA and RagA^{Q66L} were localized in the cytoplasm, but RagA$^{gtr1-11}$ was localized in the nucleus as speckles similar to COS cells expressing RagA$^{gtr1-11}$. These results suggested that the cellular localization of RagA changed depending on the bound nucleotide state.

The close similarity of the staining pattern between SC-35 and RagA$^{gtr1-11}$ suggested that RagA$^{gtr1-11}$ might be involved in mRNA splicing. However, the cytoplasmic localization of wild-type RagA in BHK21 cells did not change in the presence of actinomycin D, the concentration of which caused the cytoplasmic accumulation of A1 (data not shown) as reported (Pinol-Roma and Dreyfuss, 1992). Thus, the shuttling of RagA did not seem to be correlated with mRNA metabolism.

DISCUSSION

Using primers which are constructed according to the reported nucleotide sequence, human *RagA* and *RagB* cDNAs were amplified by PCR from the human B cell library. Whereas a single form of *RagA* was amplified, two forms of *RagB*, *RagB^{n}*

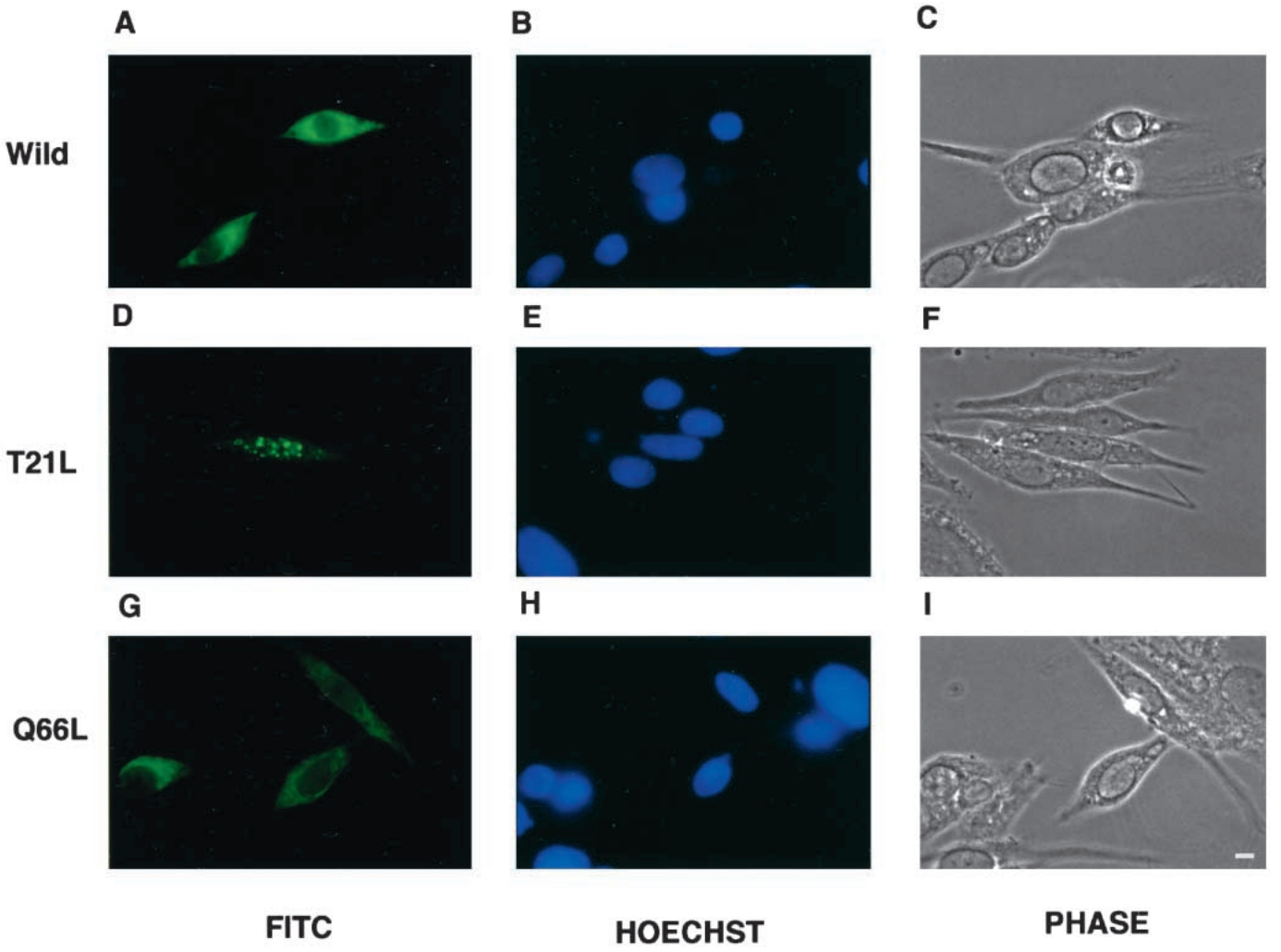

Fig. 7. Localization of RagA depending on the nucleotide bound form. 2×10^5 cells of BHK21 cell line were seeded on a slide cover glass in a 35 mm tissue culture dish. After 24 hours incubation, 2 μg/dish of each pcDEBΔ DNAs which were mixed with 6 μl of lipofectamine reagent (Gibco BRL) was introduced into cells: wild-type RagA (Wild) (A,B,C), $RagA^{gtr1\text{-}11}$ (T21L) (D,E,F), $RagA^{Q66L}$ (Q66L) (G,H,I). After incubation of 5 hours, medium was replaced with fresh one containing 10% FCS. Cells were fixed 43 hours later with methanol, doubly stained with anti-T7 antibody and then FITC-conjugated anti-mouse IgG (A,D,G) and DNA was stained with Hoechst dye (B,E,H) as described in Materials and Methods. Photographs were taken with the fluorescent microscope, and phase contrast image of cells (C,F,I). Bars, 15 μm.

and *RagB*^{s} were obtained. $RagB^s$ corresponds to the previously reported human $RagB^s$, however, $RagB^n$ is a new form of RagB. It may be produced by an alternative splicing of RagB mRNA as reported in the case of $RagB^l$ which has been thought to be produced by an alternative splicing of *RagB* mRNA (Schürman et al., 1995). Taking these previous reports together, our present results indicate that the splicing of RagB mRNA is alternative, whereas that of RagA is not.

Both *RagA* and *RagB*^{s} cDNAs rescue the cold sensitivity of *gtr1-11*, but *RagB*^{n} cDNA does not. The inability of *RagB*^{n} cDNA to rescue *gtr1-11* is consistent with previous reports showing that *S. cerevisiae gtr1-3*Δ which corresponds to *RagB*^{n} is cold sensitive for growth (Bun-ya et al., 1992; Nakashima et al., 1996). Thus, $RagB^n$ may have no biological activity at 14°C, the nonpermissive temperature for *gtr1-11*. Taking these findings together, human *RagA* and *RagB*^{s} can be concluded to rescue the cold sensitivity of *gtr1-11*, and therefore they are functional mammalian homologues of *S. cerevisiae GTR1*. The functional conservation between human RagA and Gtr1p is further confirmed in another way. Previously, we reported that the cold-sensitive mutation of *GTR1*, *gtr1-11*, suppressed both *rcc1*$^-$ and *rna1*$^-$ of *S. cerevisiae*. Furthermore, overexpression of Gtr1-11p partly, but significantly, rescues *rcc1*$^-$ mutants (Nakashima et al., 1996). Based on these findings, we introduced the *gtr1-11* mutation into the corresponding site of the human RagA. The resultant mutated human $RagA^{gtr1\text{-}11}$ partly but significantly suppressed all of the *rcc1*$^-$ alleles examined and also *rna1-1*, but not *srp1-31*, similar to *gtr1-11*. Thus, RagA and RagB are functional homologues of *S. cerevisiae* Gtr1p.

Similar to Gtr1p, human RagA and $RagB^s$ are localized in the cytoplasm of COS cells. However, a dominant negative mutated form of RagA, $RagA^{gtr1\text{-}11}$, is localized in the nucleus and is distributed in speckles similar to SC-35, a non-snRNP of the splicing complex (reviewed by Lamm and Lamond, 1993). Speckles of both $RagA^{gtr1\text{-}11}$ and SC-35 seem to be

randomly distributed in the nucleus. When speckles of RagA$^{gtr1\text{-}11}$ and SC-35 are superimposed, however, SC-35 is juxtaposed to RagA$^{gtr1\text{-}11}$. No co-immunoprecipitation of RagA$^{gtr1\text{-}11}$ with SC-35 indicates that there is no direct interaction between them.

The mutation of RagA$^{gtr1\text{-}11}$ corresponds to the GDP-bound form of Ran-GTPase mutant, T24N, which tightly binds to RCC1, so that T24N Ran is concentrated in the nucleus where RCC1 is localized (Dasso et al., 1994). Therefore, the accumulation of mutated Gtr1-11p near the splicing complexes may suggest that the GDP/GTP exchange of RagA occurs near the spliceosome to regulate mRNA maturation. In this context, we investigated whether actinomycin D, an inhibitor of RNA transcription caused an accumulation of wild-type RagA in the nucleus. But there was no clear accumulation of RagA. Interestingly, the GTP-bound form of RagA^{Q66L} is localized in the cytoplasm. While the functional relationship between RagA and SC-35 remains to be determined, thus, RagA seems to shuttle between the nucleus and the cytoplasm, depending on the bound nucleotide state. Recently, a nuclear domain designated ND10 (Maul et al., 1996), also referred to as PML (Weiss et al., 1994) is reported to be localized side by side with SC-35 (Ishov et al., 1997). ND10/PML are multiprotein complexes of the nucleus, which are upregulated by interferons (Lavau et al., 1995). The reported distribution of ND10/PML is quite similar to that of RagA$^{gtr1\text{-}11}$. We are currently investigating the relationship between ND10/PML and the speckles of RagA$^{gtr1\text{-}11}$.

We thank Drs H. Iida and K. Nakamua (Department of Anatomy, Faculty of Medicine, Kyushu University) and T. Kanemaru (Morphology core, Faculty of Medicine, Kyushu University) for taking laser-scanning microscope pictures. This work was supported by Grants-in-Aid for Specially Promoted Research and the HFSP. The English used in this manuscript was revised by Miss K. Miller (Royal English Language Centre, Fukuoka, Japan).

REFERENCES

Aebi, M., Clark, M. W., Vijayraghavan, U. and 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.

Belhumeur, P., Lee, A., Tam, R., DiPaolo, T., Fortin, N. and Clark, M. W. (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, F. R. and Ponstingl, H. (1991a). Mitotic regulator protein RCC1 is complexed with a nuclear ras-related polypeptide. *Proc. Nat. Acad. Sci. USA* **88**, 10830-10834.

Bischoff, F. R. and Ponstingl, H. (1991b). Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature* **354**, 80-82.

Bischoff, F. R., Krebber, H., Kempf, T., Hermes, I. and Ponstingl, H. (1995). Human RanGTPase-activating protein RanGAP1 is a homologue of yeast Rna1p involved in mRNA processing and transport. *Proc. Nat. Acad. Sci. USA* **92**, 1749-1753.

Boguski M. S. and McCormick, F. (1993). Proteins regulating Ras and its relatives. *Nature* **366**, 643-653.

Bun-ya, M., Harashima, S. and Oshima, Y. (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.

Chuang, R.-Y., Weaver, P. L., Liu, Z. and Chang, T.-H. (1997). Requirement of the DEAD-Box protein Ded1p for messenger RNA translation. *Science* **275**, 1468-1471.

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

Dasso, M., Seki, T., Azuma, Y., Ohba, T. and Nishimoto, T. (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.

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

Fu, X. and Maniatis, T. (1990). Factor required for mammalian splicesome assembly is localized to discrete regions in the nucleus. *Nature* **343**, 437-441.

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

Gluzman, Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutant. *Cell* **23**, 175-182.

Hayashi, N., Seino, H., Irie, K., Watanabe, M., Clark, K. L., Matsumoto, K. and Nishimoto, T. (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**, 49-156.

Hopp, T. P., Prickett, K. S., Price, V. L., Libby, R. T., March, C. J., Cerretti, D. P., Urdal, D. L. and Conlon, P. J. (1988). A short polypeptide marker sequence useful for recombinant protein identification and purification. *Biotechnology* **6**, 1204-1210.

Hopper, A. K., Banks, F. and Evangelidis, V. (1978). A yeast mutant which accumulates precursor tRNA. *Cell* **19**, 211-219.

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

Ishov, A. M., Stenberg, R. M. and Maul, G. G. (1997). Human cytomegalovirus immediate early interaction with host nuclear structures: definition of an immediate transcript environment. *J. Cell Biol.* **138**, 5-16.

Kadowaki, T., Goldfarb, D., Spitz, L. M., Tartakoff, A. M. and 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.

Lamm, G. M. and Lamond, A. I. (1993). Non-snRNP protein splicing factors. *Biochem. Biophys. Acta* **1173**, 247-265.

Lavau, C., Marchio, A., Fagioli, M., Jansen, J., Falini, B., Lebon, P. Grosveld, F., Pandolfi, p. P., Pelicci, P. G. and Dejean, A. (1995). The acute promyelocytic leukaemia-associated PML gene is induced by interferon. *Oncogene* **11**, 871-876.

Lee, A., Clark, K. L., Fleischmann, M., Aebi, M. and Clark, M. W. (1994). Site-directed mutagenesis of the yeast *PRP20/SRM1* gene reveals distinct activity domains in the protein product. *Mol. Gen. Genet.* **245**, 32-44.

Makishima T., Nakashima, T., Nagata-Kuno, K., Fukushima, K., Iida, H., Sakaguchi, M., Ikehara, Y., Komiyama, S. and Nishimoto, T. (1997). The highly conserved DAD1 protein involved in apoptosis is required for N-linked glycosylation. *Genes to Cells* **2**, 129-141.

Matsumoto, T. and Beach, D. (1991). Premature initiation of mitosis in yeast lacking RCC1 or an interacting GTPase. *Cell* **66**, 347-360.

Maul, G. G., Ishov, A. M. and Everett, R. D. (1996). Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. *Virology* **217**, 67-75.

Michael, S. F. (1994). Mutagenesis by incorporation of a phosphorylated oligo during PCR amplification. *BioTechniques* **16**, 410.

Misteli, T. and Spector, D. L. (1997). Protein phosphorylation and the nuclear organization of pre-mRNA splicing. *Trends Cell Biol.* **7**, 135-138.

Nakashima, N., Hayashi, N., Noguchi, E. and Nishimoto, T. (1996). Putative GTPase Gtr1p genetically interacts with the RanGTPase cycle in Saccharomyces cerevisiae. *J. Cell Sci.* **109**, 2311-2318.

Nakashima, T., Sekiguchi, T., Kuraoka, A., Fukushima, K., Shibata, Y., Komiyama, S. and Nishimoto, T. (1993). Molecular cloning of a human cDNA encoding a novel protein, DAD1 whose defect causes apoptotic cell death in hamster BHK21 cells. *Mol. Cell. Biol.* **13**, 6367-6374.

Nishimoto, T., Eilen, E. and Basilico, C. (1978). Premature chromosome condensation in a ts DNA-mutant of BHK cells. *Cell* **15**, 475-483.

Ninomiya-Tsuji, J., Nomoto, S., Yasuda, H., Reed, S. I. and Matsumoto, K. (1991). Cloning of a human cDNA encoding a CDC2-related kinase by complementation of a budding yeast cdc28 mutation. *Proc. Nat. Acad. Sci. USA* **88**, 9006-9010.

Noguchi, E., Hayashi, N., Nakashima, N. and Nishimoto, T. (1997). Yrb2p,

Nup2p-related yeast protein has functional overlap with Rna1p, yeast RanGAP protein. *Mol. Cell Biol*. **17**, 2235-2246.

Ohtsubo, M., Kai, R., Furuno, N., Sekiguchi, T., Sekiguchi, M., Hayashida, H., Kuma, K., Miyata, T., Fukushige, S., Murotu, T., Matsubara, K. and 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.

Pinol-Roma, S. and Dreyfuss, G. (1992). Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature* **355**, 730-732.

Ren, M., Drivas, G., D'Eustachio, P. and Rush, M. G. (1993). Ran/TC4: a small nuclear GTP-binding protein that regulates DNA synthesis. *J. Cell Biol*. **120**, 313-323.

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

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

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

Schmid, S. R and Linder, P. (1992). D-E-A-D protein family of putative RNA helicases. *Mol. Microbiol*. **6**, 283-292.

Schürmann, A., Brauers, A., Maßmann, S., Becker, W. and Joost, H-G. (1995). Cloning of a novel family of mammalian GTP-binding proteins (RagA, RagB^s, RagB^1) with remote similarity to the Ras-related GTPase. *J. Biol. Chem.* **270**, 28982-28988.

Weiss, K., Rambaud, S., Lavau, C., Jansen, J., Carvalho, T., Carmo-Fonseca, M., Lamond, A. and Dejean, A. (1994). Retinoic acid regulates aberrant nuclear localization of PML-RAR a in acute promyelocytic leukemia cells. *Cell* **76**, 345-356.

Xing, Y., Johnson, C. V., Moen, P. T. Jr, McNeil, J. A. and Lawrence, J. B. (1993). Nonrandom gene organization: Structural arrangements of specific pre-mRNA transcription and splicing with SC-35 domains. *J. Cell Biol.* **131**, 1635-1647.

Yano, R., Oakes, M. L., Tabb, M. M. and Nomura, M. (1994). Yeast Srp1p has homology to armadillo/plakoglobin/β-catenin and participates in apparently multiple nuclear functions including the maintenance of the nucleolar structure. *Proc. Nat. Acad. Sci. USA* **91**, 6880-6884.