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Human Dis3p, Which Binds to Either GTP- or GDP-Ran, Complements *Saccharomyces cerevisiae dis3*[1]

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***Saccharomyces cerevisiae* Dis3p, which interacts with Ran/Gsp1p, complements *Schizosaccharomyces pombe dis3-54*. Consistent with the functional conservation of Dis3p in *S. cerevisiae* and *S. pombe*, the human ORF (accession number: R27667) was found to be highly homologous to yeast Dis3p. Based on its nucleotide sequence, we cloned a full-sized human *DIS3* cDNA. The cloned human cDNA partly but significantly restored the temperature-sensitivity of *S. cerevisiae dis3*. Thus, Dis3p was found to be structurally and functionally conserved from yeast to mammals. Consistent with the report that *S. cerevisiae* Dis3p is identical to Rrp44p, which comprises the exosome involved in ribosomal RNA processing, *S. cerevisiae* Dis3p was found to be localized in the nucleolus. Similar to *S. cerevisiae* Dis3p, human Dis3p enhanced RCC1-stimulated nucleotide release from Ran, in a dose-dependent manner, and bound to GTP- or GDP-Ran.**

Key words: Dis3, Gsp1p, Ran, Rrp44, rRNA.

Ran is a nuclear Ras-like GTPase required for the nuclear pore transport function (*1*). The phenotype of *rcc1*, a temperature-sensitive (ts) mutant of Rcc1p, the GDP/GTP exchanging factor for Ran, however, is pleiotropic (*2, 3*), suggesting that Ran may function in nuclear processing in addition to nuclear protein import and mRNA export (*1*). *Saccharomyces cerevisiae* Ran homologue Gsp1p/Cnr1p has been cloned as a multi-copy suppressor of *S. cerevisiae rcc1* alleles, *prp20-1* and *mtr1-2* (*4, 5*). *S. cerevisiae rcc1* can be complemented by mammalian *RCC1* and *vice versa* (*6, 7*). Thus, the Ran GTPase cycle has been functionally conserved throughout evolution.

Through analogy with the Ras family, proteins which specifically bind GTP-Ran will be effectors for Ran (*8*). In order to clarify the functions of Ran, proteins which specifically interact with GTP-Ran have been identified either biochemically or genetically. They comprise a family of proteins possessing a domain homologous to RanBP1 (*3, 9*) and importin β (*10*). In contrast to these GTP-Ran binding proteins, p10/NTF2 specifically binds to GDP-Ran (*11*). The finding that overexpression of Ntf2p rescues temperature-sensitive mutations of Gsp1p (*12*) indicates a tight interaction between Ran/Gsp1p and p10/Ntf2p, while the role of p10/Ntf2p in the Ran GTPase cycle is known yet. Previously, we isolated *S. cerevisiae* Dis3p by means of two-hybrid screening of a *S. cerevisiae* genomic DNA library using human Ran as a bait (*13*). While the Ran-binding proteins thus far mentioned specifically bind GTP- or GDP-Ran, *S. cerevisiae* Dis3sc is suggested to bind a nucleotide-free Ran/Gsp1p, thereby, enhances the RCC1-stimulated nucleotide release from Ran (*13*).

Dis3p was originally identified as a protein defective in cold-sensitive *Schizosaccharomyces pombe* mutant *dis3* (*14*). At the same time, *dis1* and *dis2* were isolated. These mutants are mutually synthetic lethal. Dis2p is the catalytic subunit of a type 1 protein phosphatase (*15, 16*). Recently, Dis3sc was reported to be identical to Rrp44p, which comprises the exosome exhibiting 3′ to 5′ exoribonuclease activity, with other Rrp4, Rrp41, Rrp42, and Rrp43 (*17*). Thus, Dis3p is suggested to be involved in the 3′ processing of 5.8S rRNA. The human homologue of Rrp4 rescues the *S. cerevisiae rrp4-1* mutation (*17*). Similar to hRrp4, Dis3hs/hRrp44p rescued the temperature-sensitivity of *S. cerevisiae dis3*. Interestingly, human Dis3p bound both GTP- and GDP-Ran.

MATERIALS AND METHODS

Strains and Media—The *S. cerevisiae* strains and plasmids used in this study are described in Table I. The *S. cerevisiae* strains were transformed by a modified LiCl method involving DMSO, as described (*13*). *Escherichia coli* strains XLI-blue and BL21(DE3) were used for plasmid-engineering and producing recombinant proteins as described previously (*18*). The media used respectively for yeasts and bacteria were described previously (*19*).

Amplification of Human DIS3 cDNA-Fragments—According to the nucleotide sequence of human cDNA (Gen-

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Abbreviations: mAb, monoclonal antibody; GST, glutathione S-transferase; GEF, guanine nucleotide exchanging factor.

TABLE I. **Plasmids and yeast strains used in this study.**

Plasmid	Relevant markers	Description	Source or reference
pGAP-DIS3hs	*CEN TRR1* P_{TDH3}-*DIS3hs*	pGAP314[a] with *DIS3hs* cDNA inserted downstream of *TDH3* promoter	This study
pGAP-DIS3sc	*CEN TRR1* P_{TDH3}-*DIS3sc*	pGAP314[a] with *DIS3sc* cDNA inserted downstream of *TDH3* promoter	This study
pGAP-GSTDIS3sc	*CEN TRP1* P_{TDH3}-*GSTDIS3sc*	pGAP314[a] with *GST-DIS3sc* cDNA inserted downstream of *TDH3* promoter	This study
pEG-KG-DIS3hs	*2μ URA3 Leu2-d* P_{GAL10}-*GSTDIS3hs*	pEG-KG[b] with *GST-DIS3hs* cDNA inserted downstream of *GAL10* promoter	This study

Yeast	Genotype	Source or reference
N43	*MATa/α ade2/ade2 his3/his3 leu2/leu2 LYS2/lys2 trp1/trp1 ura3/ura3*	*13*
YPH501	*MATa/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3*	*33*
37C19	*MATα dis3::HIS3 ade2 his3 leu2 lys2 trp1 ura3* [YCpDIS3sc]	*13*
37C19-A	*MATα dis3::HIS3 ade2 his3 leu2 lys2 trp1 ura3* [pGAP-GSTDIS3sc]	37C19 plasmid shuffled with pGAP-GSTDISA3sc
dis3-81	*MATα dis3-81 ade2 his3 leu2 trp1 ura3 can1*	NBW5[c] replaced with *dis3-81* temperature-sensitive allele (Noguchi *et al.*, in preparation)

[a]pGAP314 in Ref. *19*. [b]pEG-KG in Ref. *21*. [c]NBW5 in Ref. *19*.

Bank accession number: R27667), 5′ and 3′ primers for PCR, 5′-TTGTTGCTATTGATGGTTGGCC and 3′-CCTGGTGGGTCTACACTACAA, were prepared, respectively. Using these primers, human cDNA fragments were amplified from mRNA of HeLa cells by 40 cycles of reactions; 94°C for 45 s, 55°C for 60 s, and 72°C for 60 s, using Taq polymerase. The 5′ stretch cDNA library of a human B cell lymphoma (Clontech) was then screened using the resultant human cDNA as a probe, as described (*20*).

Construction of GST-Fused Human DIS cDNA—To remove the stop codon prior to the first methionine of human Dis3p, obtained human *DIS3* cDNA clone No. 21 was amplified with KOD polymerase (Toyobo) for 30 cycles of reactions; 98°C for 15 s, 65°C for 2 s, and 72°C for 30 s, using as the 5′ and 3′ primers, 5′-GCGGGATCCGGCAAGATGCTCAAGTCCAAG and 3′-ACCACAGAAGATGGTGC, respectively. The amplified DNA fragments were digested with restriction enzymes, *Bam*HI and *Nhe*I, and then used to replace the *Bam*HI-*Nhe*I fragment of the originally cloned human *DIS3* cDNA. From the resultant plasmid, 2.9 kb of *DIS3hs* was cut out with *Bam*HI and *Xho*I, and then inserted into the *Bam*HI/*Xho*I sites of pET32a (Novagen). From the resultant plasmid, *DIS3hs* was then cut out with *Nco*I and *Xho*I, and then inserted into the *Nco*I/*Xho*I sites of pGEX-KG, resulting in *GST*-fused *DIS3hs*. From the resultant plasmid, *DIS3hs* was cut out with *Xba*I and *Xho*I, and then inserted into the *Xba*I/*Sal*I sites of pEG-KG (*21*).

Purification of Human Dis3p—S. cerevisiae strain NBW5/pEG(KG)-DIS3hs was cultivated up to $A_{660}=2.0$, and then for another 2 h in the presence of galactose (final, 2%), at 30°C. The cells were spun down, suspended in lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, and 1 mM DTT), lysed by vortexing with glass beads, and then centrifuged at 28,000 rpm for 1 h. From the supernatant, GST-fused human Dis3p was purified on a glutathione column as described (*13*). When indicated, GST was cut out of GST-Dis3hs by incubation in the presence of thrombin (1% w/w) at 30°C for 2 h as described (*13*).

Indirect Immunofluorescence Staining—Cultures of *S. cerevisiae* were processed and fixed as described (*22*). Cells were stained with polyclonal antibodies to *S. cerevisiae*

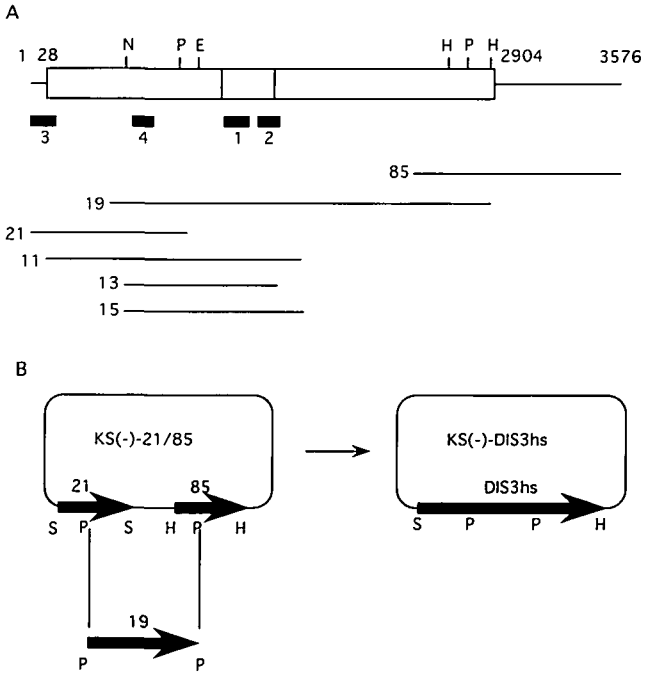

Fig. 1. **Molecular cloning of human *DIS3*.** A: Composite restriction enzyme map of human *DIS3*. Six cDNA clones were isolated and mapped on the composite human *DIS3* cDNA. The 5′ end, designated as 1 corresponds to the first nucleotide of the No. 21 clone. The ORF (open box) of human Dis3p comprises 28th to 2,904 nucleotides. "3576" corresponds to the 3′ end of the No. 85 clone. N, *Nhe*I; P, *Pfl*MI; E, *Eco*RI; H, *Hin*dIII; S, *Sma*I. The stippled box indicates the position of human ORF R27667, and the black boxes the positions of the PCR primers used. B: Construction of the full sized human *DIS3*. cDNA fragments 21 and 85 were inserted into the *Sma*I and *Hin*cII sites of the same Bluescript II KS(−), digested with *Pfl*MI, and then ligated with the *Pfl*MI fragment of cDNA fragment 19.

Dis3p, which were prepared in rabbits using as an antigen the N-terminal part (30 to 274 amino acids) of *S. cerevisiae* Dis3p, that was produced in *E. coli*. When indicated, cells were doubly stained with a monoclonal antibody (mAb), YN2C1, to *S. cerevisiae* nucleolar protein Ssb1p (*23*) (a gift from Dr. M. Nomura) and the anti-*S. cerevisiae* Dis3p antibodies. Cells that had been exposed to the primary antibodies were treated with the secondary antibodies,

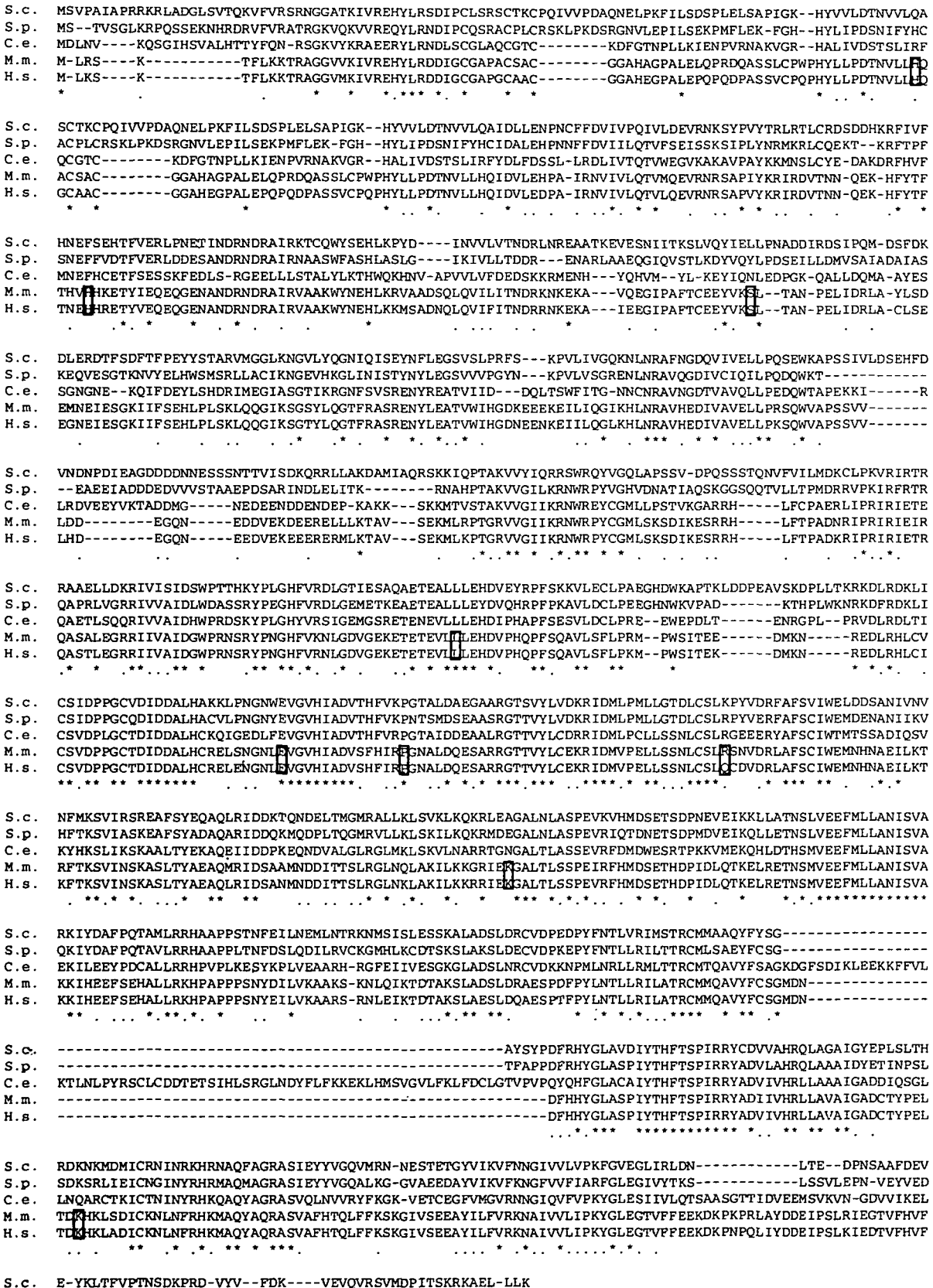

Fig. 2. **Alignment of Dis3p.** The amino acid sequence of human Dis3p presently obtained was aligned with those of *S. cerevisiae* (S. c), *S. pombe* (S. p), *Caenorhabditis elegans* (C. e), and *Mus musculus* (M. m) Dis3p, the GenBank accession numbers of which are YSPDIS3P, D76430, CEC04G2_6, and prf: 2311380A, respectively. The nucleotide sequence data for human (*Homo sapiens*, H. s) *DIS3* reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number, AB001743. The amino acids in squares were found to differ from those of human Dis3p reported by Lim *et al.* (*25*).

rhodamine-conjugated goat anti-mouse antibodies (CAPPEL) and FITC-conjugated goat anti-rabbit IgG antibodies (TAGO). Cells were finally stained with Hoechst 33342 and then mounted on Vectashield (Vector).

Zeiss Axio Photo was used for sample analysis by the standard microscopic method. Digital imaging of stained cells was performed with a Zeiss confocal laser scan microscope, LSM310.

Guanine Nucleotide Release Assay—E. coli produced human Ran and Rcc1p were purified and [^{3}H]GDP-Ran was prepared, as described (*2, 13*). Two pmol of [^{3}H]GDP-Ran was mixed with human Dis3p in GEF buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT, 1 mM CHAPS, and 2 mM GDP). After incubation at 30°C for 3 min, 20 fmol of Rcc1p was added and the reaction was carried out as described (*13*). The radioactivity remaining on Ran was counted with a liquid scintillation counter.

Biosensor Analysis—Real-time interaction analysis of the binding between human Dis3p and Ran was performed with a BIAcore biosensor instrument (Pharmacia Biosensor AB) as described (*24*). CM5 sensor chips and amine coupling kits were obtained from Pharmacia Biosensor AB. The mAb to GST (Pharmacia Biosensor AB) was immobilized on the CM5 sensor chip of the BIA core as recommended by the manufacturers, using HBS buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 0.005% Tween 20) as the running buffer. GST-RanGMPPNP, GST-RanGDP and, as a control, glutathione S-transferase were trapped.

Binding experiments were performed with injection of increasing amounts (μM) of human Dis3p in buffer comprising 20 mM potassium phosphate (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$, and 0.005% Tween 20. Evaluation and calculation of the binding parameters were carried out according to the manual BIA evaluation software type 1 model provided by Pharmacia Biosensor AB.

RESULTS

Cloning of Human DIS3 cDNA—The finding that Dis3p is structurally and functionally well conserved in *S. cerevisiae* and *S. pombe* (*13*) indicates that Dis3p plays an important role in cell proliferation. Consistent with this notion, we found that there is a human cDNA fragment (accession number: R27667) of which the amino acid sequence is highly homologous to that of yeast Dis3p. In order to prove the functional conservation of Dis3p throughout evolution, we isolated the full sized human Dis3p.

First, we amplified cDNA from mRNA of HeLa cells using as primers nucleotides prepared according to the sequence of cDNA R27667 (Fig. 1A, primers 1 and 2). The resultant human cDNA fragments were used as probes for the screening of the cDNA library of a human B cell lymphoma (Clontech) as described (*20*). Out of the 400,000 plaques screened, three cDNA fragments, Nos. 11, 13, and 15, were obtained. Using No. 11 cDNA as a probe, 600,000 plaques of the cDNA library were screened and two positive clones, Nos. 19 and 21 were obtained. Finally, using the C-terminal fragment of cDNA clone No. 19 as a probe, we obtained the No. 85 cDNA clone out of 600,000 plaques. The cDNA fragments obtained were mapped in the open reading frame of human *DIS3* cDNA (Fig. 1A).

A linear cDNA encoding the whole human Dis3p was constructed as follows. First, cDNA fragment Nos. 21 and 85 were inserted into the *Sma*I and *Hinc*II sites of the same Bluscript II KS(−) plasmid (Stratagene), respectively, resulting in plasmid KS (−)-21/85 (Fig. 1B). Both human *DIS3* cDNA fragments, Nos. 21 and 85, were found to possess a single restriction enzyme site, *Pfl*MI (P in Fig. 1A), so the resultant plasmid DNA was digested with *Pfl*MI in order to remove the intermediate nucleotide between the No. 21 and 85 regions of KS (−)-21/85, and then ligated with 1.5 kb of the *Pfl*MI fragment of the No. 19 cDNA, as shown in Fig. 1B.

The resultant human *DIS3* cDNA possessed an open reading frame of 2,874 nucleotide pairs. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number, AB001743. After cloning of the full sized human *DIS3* cDNA, Lim *et al.* (*25*) reported the sequences of full sized murine and human *DIS3* cDNA. The amino acid sequence estimated from our cDNA clone was mostly identical to the sequence of the reported human Dis3p, except for the 75th, 127th, 200th, 434th, 501th, 515th, 552th, 627th, and 807th amino acid residues (Fig. 2). In comparison with the sequence of the reported murine Dis3p, the estimated amino acid sequence of the presently cloned *DIS3hs* was found to be identical with that of the murine Dis3p, except for the 552th amino acid residue. Based on our sequence, human Dis3p is 90.4% identical to the murine one.

Human Dis3p Is a Functional Homologue of S. cerevisiae Dis3p—In order to clarify the functional conserva-

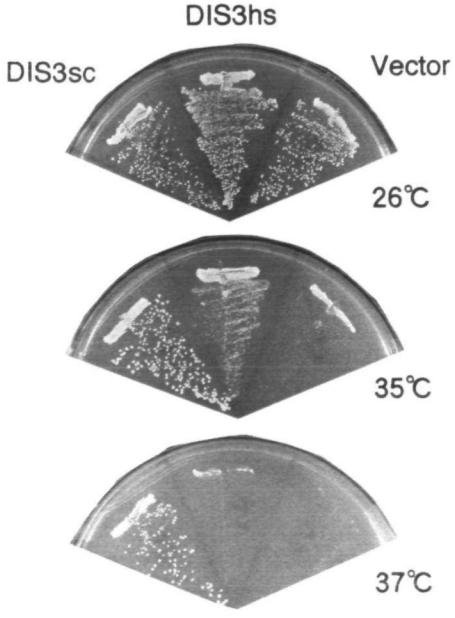

Fig. 3. **Human *DIS3* complements *S. cerevisiae dis3*.** *DIS3hs* (Dis3hs) and *DIS3sc* (Dis3sc) carried on *S. cerevisiae* expression vector pGAP314, and the vector alone (Vector) were introduced into *S. cerevisiae* strain *dis3-81* by a modified LiCl method involving DMSO. Transformants were selected on synthetic medium plates (*trp*$^-$), streaked onto synthetic medium plates (*trp*$^-$), and then incubated for 4 days at the indicated temperatures.

tion in human and *S. cerevisiae* Dis3p, the human *DIS3* cDNA was inserted into the *S. cerevisiae* expression vector, pGAP314 (*TRP1, CEN, TDH3* promoter) (*22*). The resultant pGAP-DIS3hs was introduced into a temperature-sensitive mutant of *S. cerevisiae* strain NBW5, *dis3-81*, in which the *DIS3* gene was replaced by the *dis3-81* allele (Noguchi *et al.*, in preparation). As controls, pGAP-DIS3sc and the vector alone were introduced into *dis3-81*, as well.

Transformants expressing human *DIS3* were selected in medium lacking tryptophan, and then cultured at 26, 35, or 37°C as indicated (Fig. 3). *dis3-81* cells grew at 26°C, but not at 35 or 37°C (Fig. 3, vector). Transformants expressing human *DIS3* grew at 35°C, but not at 37°C, while *S. cerevisiae DIS3* rescued the growth defect of *dis3-81* even at 37°C. These results indicated that the function of human Dis3p partially but significantly overlapps that of *S. cervisiae* Dis3p.

Localization of Dis3p—Previously, we found that *S. pombe* Dis3p forms a complex with *S. pombe* RCC1 homologue Pim1p, which is located on chromatin (*13, 26, 27*). Consistent with this fact, *S. pombe* Dis3p is enriched in the nuclear fraction (*13*). In order to determine the cellular localization of *S. cerevisiae* Dis3p, we prepared antibodies to *E. coli*-produced Dis3sc fragment. The anti-Dis3sc antibodies prepared recognized proteins of the same molecular mass in strain 37C19 {*⊿dis3* [YCpDIS3sc]} as in endogenous Dis3p (Fig. 4A, compare lanes 1 and 3), but recognized proteins of 140 kDa corresponding to GST-fused Dis3sc in strain 37C19-A {*⊿dis3* [pGAP-GSTDIS-3sc]} (Fig. 4A, lane 2). Thus, the anti-Dis3sc antibodies prepared specifically recognized *S. cerevisiae* Dis3p.

Exponentially growing cultures of *S. cerevisiae* N43 were fixed and stained with the anti-Dis3sc antibodies. DNA was stained with Hoechst 33342. As shown in Fig. 4B, the

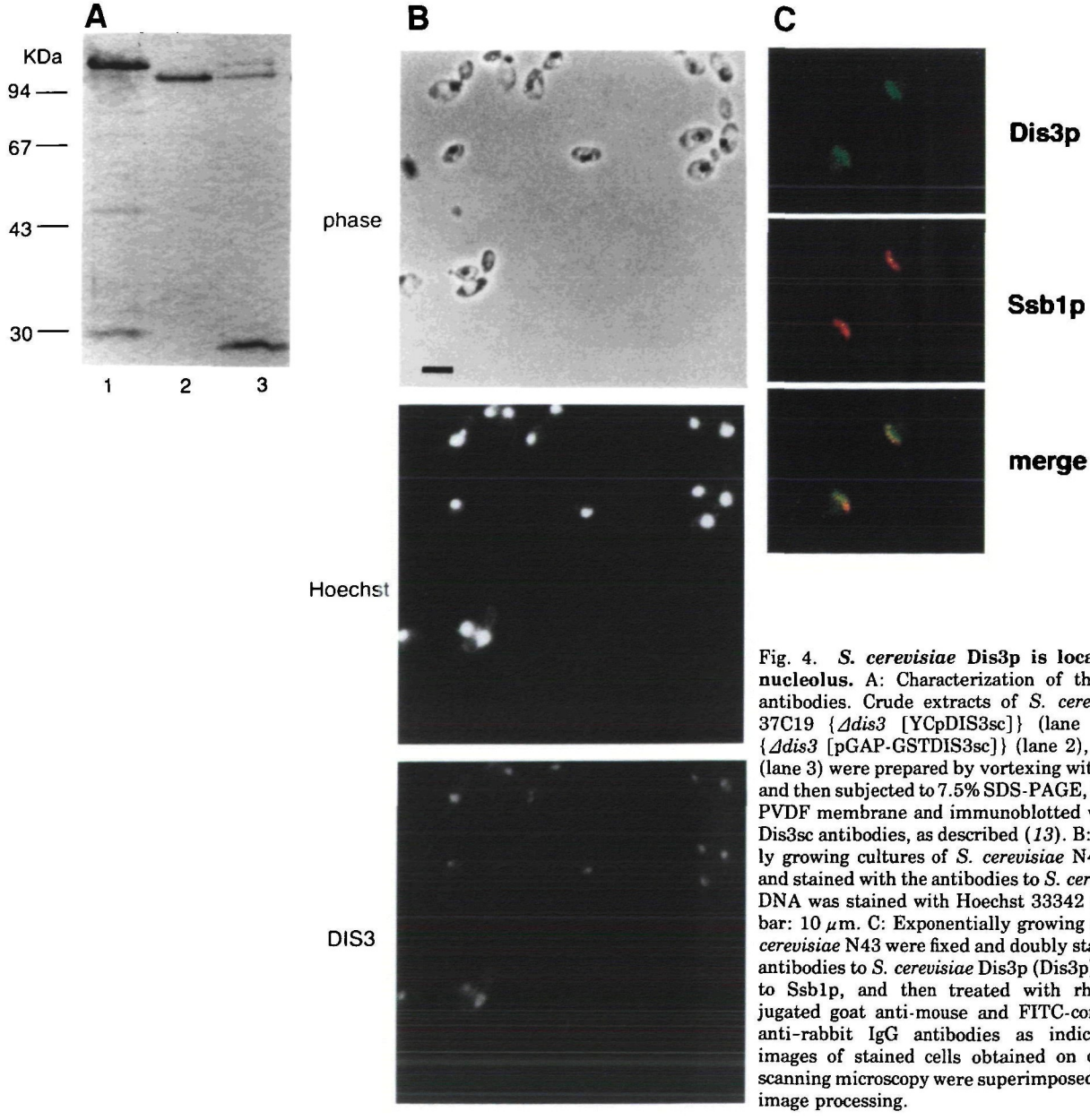

Fig. 4. ***S. cerevisiae* Dis3p is localized in the nucleolus.** A: Characterization of the anti-Dis3sc antibodies. Crude extracts of *S. cerevisiae* strains 37C19 {*⊿dis3* [YCpDIS3sc]} (lane 1), 37C19-A {*⊿dis3* [pGAP-GSTDIS3sc]} (lane 2), and YPH501 (lane 3) were prepared by vortexing with glass beads, and then subjected to 7.5% SDS-PAGE, transferred to PVDF membrane and immunoblotted with the anti-Dis3sc antibodies, as described (*13*). B: Exponentially growing cultures of *S. cerevisiae* N43 were fixed and stained with the antibodies to *S. cerevisiae* Dis3p. DNA was stained with Hoechst 33342 (DNA). Scale bar: 10 μm. C: Exponentially growing cultures of *S. cerevisiae* N43 were fixed and doubly stained with the antibodies to *S. cerevisiae* Dis3p (Dis3p) and the mAb to Ssb1p, and then treated with rhodamine-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit IgG antibodies as indicated. Digital images of stained cells obtained on confocal laser scanning microscopy were superimposed by electronic image processing.

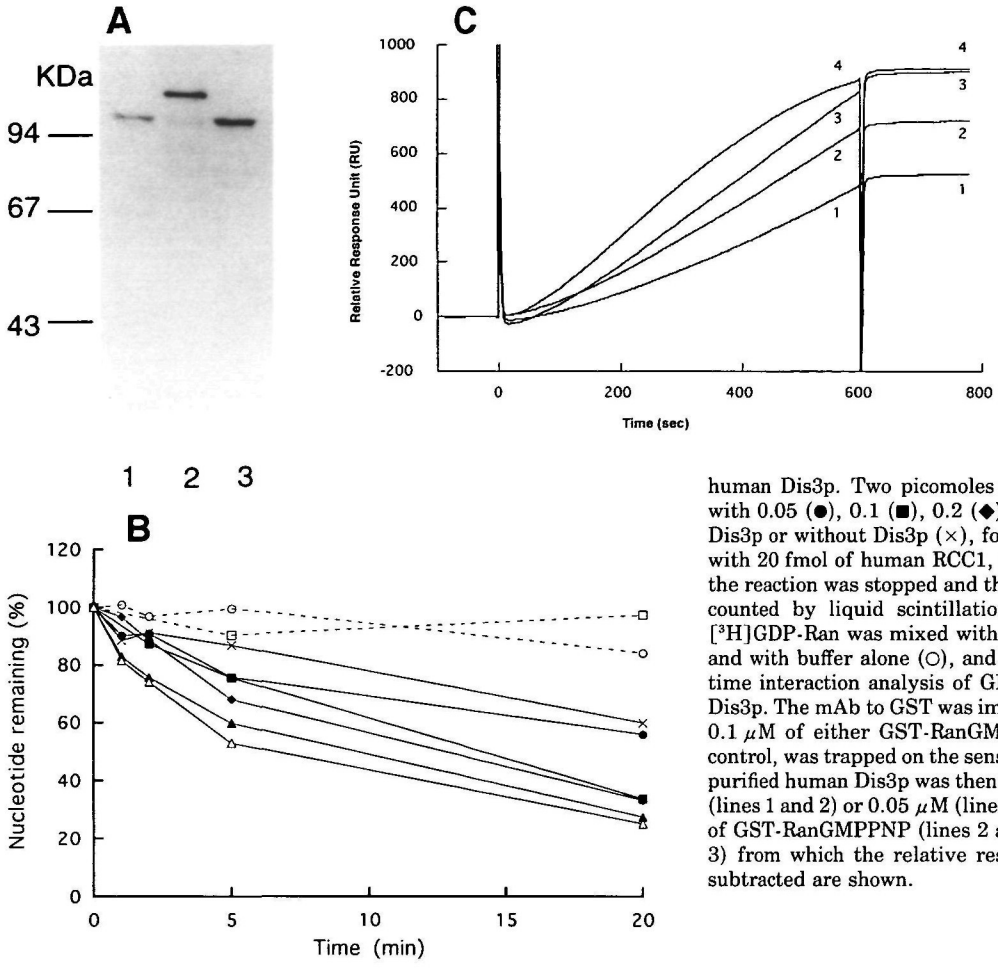

Fig. 5. **Dis3p binds to both GDP- and GTP-Ran.** A: SDS-polyacrylamide gel electrophoretic analysis of purified GST-fused human Dis3p. About 80 μg of GST-fused human Dis3p was digested with thrombin and then the beads were spun down. GST-fused human Dis3p, and proteins in the supernatant and on the beads were electrophoresed on SDS-polyacrylamide (10%) gels and then stained with Coomassie Brilliant Blue. Lane 1: GST-human Dis3p. Lane 2: human Dis3p digested with thrombin. Lane 3: GST-beads. B: Enhancement of RCC1-stimulated nucleotide release by human Dis3p. Two picomoles of [^{3}H]GDP-Ran was pre-incubated with 0.05 (●), 0.1 (■), 0.2 (◆), 0.4 (▲), or 1.0 pmol (△) of human Dis3p or without Dis3p (×), for 3 min at 30°C, and was then mixed with 20 fmol of human RCC1, as indicated. At the indicated times, the reaction was stopped and the remaining radioactivity on Ran was counted by liquid scintillation counting. As controls, 2 pmol of [^{3}H]GDP-Ran was mixed with 1.0 pmol of human Dis3p alone (□), and with buffer alone (○), and then incubated as indicated. C: Real time interaction analysis of GDP- and GTP-Ran binding to human Dis3p. The mAb to GST was immobilized on the sensor chip and then 0.1 μM of either GST-RanGMPPNP, GST-RanGDP, or GST, as a control, was trapped on the sensor chip through the mAb to GST. The purified human Dis3p was then injected at the concentration of 0.025 (lines 1 and 2) or 0.05 μM (lines 3 and 4). The relative response units of GST-RanGMPPNP (lines 2 and 4) and GST-RanGDP (lines 1 and 3) from which the relative response units of GST alone had been subtracted are shown.

staining pattern of Dis3p appeared to be that of the nucleolus. In order to prove this, cells were doubly stained with the anti-Dis3sc antibodies and, as a nuclolus-specific probe, with the monoclonal antibody (mAb) to *S. cerevisiae* nucleolar protein Ssb1p (a single-stranded nucleic acid-binding protein) (*23*). When superimposed, the staining patterns of Ssb1p and Dis3p well overlapped (Fig. 4C). Thus, Dis3p is localized in the nucleolus, consistent with the report of Rad *et al.* (*28*).

Human Dis3p Binds Both GTP- and GDP-Ran—Previously, we found that *S. cerevisiae* Dis3p could enhance RCC1-stimulated nucleotide release (*13*). In order to confirm that this finding is also true for human Dis3p, we expressed human Dis3p in *S. cerevisiae* and purified it on a glutathione column. The purified GST-fused human Dis3p was finally digested with thrombin to obtain GST-free human Dis3p (Fig. 5A, lane 2). Increasing amounts of the purified human Dis3p were then mixed with [^{3}H]GDP-Ran, followed by incubation with Rcc1p. The radioactivity remaining on Ran was counted with a liquid scintillation counter. As previously reported (*13*), human Dis3p enhanced RCC1-stimulated nucleotide release from GDP-Ran in a dose-dependent manner (Fig. 5B). Since we could not obtain a large amount of the purified human Dis3p, the kinetic parameters could not be calculated. Instead, we used [^{3}H]GDP-Ran containing a lot of cold GDT-Ran. Under such conditions, the nucleotide-exchange activity of Rcc1p could be reduced, so the enhancement of RCC1-stimulated nucleotide exchange with Dis3p was clearly observed. Since human Dis3p by itself did not release [^{3}H]GDP from Ran, even at the concentration of 1.0 pmol, which strongly enhanced the GEF activity of Rcc1p (Fig. 5B), our human Dis3p preparation did not contain detectable activity of *S. cerevisiae* RCC1 homologue Prp20p.

Upon the mixing of GST-fused *S. cerevisiae* Dis3p with guanine nucleotide-bound Ran, nucleotide-free Ran was co-precipitated with GST fused Dis3p (*13*), although *S. cerevisiae* or human Dis3p by itself has no ability to release nucleotides from Ran (*13* and Fig. 5B, Dis3p alone). This finding suggested that Dis3p first bound to the nucleotide-bound Ran and then the nucleotide was released from Ran during sedimentation with beads. In order to determine whether or not the nucleotide-bound Ran can directly bind human Dis3p, we analyzed the interaction between human Dis3p and the nucleotide-bound Ran by real time interaction analysis using BIAcore (*24*).

The mAb to GST was immobilized on the sensor chip of the BIA core to trap GST-fused Ran-GMPPNP, Ran-GDP, or GST alone. The interaction of human Dis3p with nucleotide-bound Ran was then determined by injecting increasing amounts (μM) of human Dis3p. The relative response units obtained with GST alone were subtracted as the background from those of GST-fused Ran. As shown in Fig. 5C, both GTP- and GDP-Ran significantly bound to both GMPPNP- and GDP-Ran. The calculated affinity constants of Ran-GMPPNP and Ran-GDP were 1.9×10^9

(M^{-1}) and 3.4×10^8 (M^{-1}), respectively. Thus, Dis3p was indicated to be able to bind to both GTP- and GDP-Ran, while GTP-Ran binds to Dis3p more efficiently.

DISCUSSION

Previously, we obtained the C-terminal fragment of *S. cerevisiae DIS3* by means of the two-hybrid method using human Ran as a bait, and subsequently cloned the full-sized ORF of *S. cerevisiae* Dis3p (*13*). *S. cerevisiae DIS3* obtained complements *S. pombe dis3*, indicating that both *S. cerevisiae* and *S. pombe* Dis3p are functionally conserved. In this study, we have cloned human *DIS3* cDNA. The encoded human Dis3p is highly homologous to *S. cerevisiae* and *S. pombe* Dis3p, as reported (*25*), and was found to be able to complement *S. cerevisiae dis3*. Thus, Dis3p is structurally and functionally conserved throughout evolution.

In both *S. pombe* and *S. cerevisiae*, Dis3p comprises a large molecular complex (*13, 16*). Recently, Dis3p was shown to be identical to Rrp44p, which comprises the exosome with Rrp4p, Rrp41p, Rrp42p, and Rrp43p (*17*). The exosome possessing the multiple 3′-5′ exonuclease activities is supposed to be required for the 3′ processing of 5.8S rRNA, which occurs in the nucleolus. Consistent with this finding, *S. cerevisiae* Dis3p is localized in the nucleolus.

Taken together with the previous report that the human homologue of *S. cerevisiae* Rrp4p, which comprises the exosome, complements the *S. cerevisiae rrp4-1* mutation (*17*), the present results indicate that the function of the exosome is conserved throughout evolution. One of the major phenotypes observed in *rcc1* is a defect in RNA processing (*2, 3, 29*). Indeed, *rna1-1*, a ts mutant of RanGTPase-activating protein Rna1p, was isolated as a mutant defective in ribosomal RNA processing (*30-32*). Through analogy with the Ras family (*8*), the function of Ran may be carried out by proteins to which GTP-Ran specifically binds. In this regard, our present finding that both GDP- and GTP-Ran bind human Dis3p is striking. This may indicate that Dis3p does not function as an effector for Ran. The other question raised by the finding that *S. cerevisiae* Dis3p is identical to Rrp44p (*18*) is whether or not the activity of the exosome is regulated by Ran. In this regard, it could be possible that the exosome, as a whole complex, binds to GTP-, but not GDP-Ran, through Rrp44p/Dis3p. While this is an attractive idea, the findings that Rrp44p/Dis3p has exoribonuclease activity (*18*) and that Dis3p enhances the nucleotide exchanging activity of Rcc1p (*13*) suggest another possibility that the role of Dis3p in the Ran GTPase cycle differs from that in the RNA processing pathway. In order to clarify this issue, we are currently isolating a series of ts mutants of *S. cerevisiae* Dis3p.

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REFERENCES

1. Nigg, E.A. (1997) Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature* **386**, 779-787
2. Dasso, M., Seki, T., Azuma, Y., Ohba, T., and Nishimoto, T. (1994) A mutant form of 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
3. Seki, T., Hayashi, N., and Nishimoto, T. (1996) RCC1 in the Ran pathway. *J. Biochem.* **120**, 207-214
4. 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 super family. *EMBO J.* **12**, 2929-2937
5. 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
6. Fleischmann, M., Clark, M.W., Forrester, W., Wickens, M., Nishimoto T., and Aebi, M. (1991) Analysis of yeast *prp20* mutations and functional complementation by the human homologue RCC1, a protein involved in the control of chromosome condensation. *Mol. Gen. Genet.* **227**, 417-423
7. Ohtsubo, M., Yoshida, T., Seino, H., Nishitani, H., Clark, K., Sprague, G., Frasch, M., and Nishimoto, T. (1991) Mutation of the hamster cell cycle gene RCC1 is complemented by the homologous genes of *Drosophila* and *S. cerevisiae*. *EMBO J.* **10**, 1265-1273
8. Boguski, M. and McCormick, F. (1993) Proteins regulating Ras and its relatives. *Nature* **366**, 643-653
9. Dingwall, C.D., Kandels-Lewis, S., and Seraphin, B. (1995) A family of Ran binding proteins that includes nucleoporins. *Proc. Natl. Acad. Sci. USA* **92**, 7525-7529
10. Gorlich, D., Dabrowski, M., Bischoff, F.R., Kutay, U., Bork, P., Hartmann, E., Prehn, S., and Izaurralde, E. (1997) A novel class of RanGTP binding proteins. *J. Cell Biol.* **138**, 65-80
11. Nehrbass, U. and Blobel, G. (1996) Role of the nuclear transport factor p10 in nuclear import. *Science* **272**, 120-122
12. Wong, D.H., Corbett, A.H., Kent, H.M., Stewart, M., and Silver, P.A. (1997) Interaction between the small GTPase Ran/Gsp1p and Ntf2p is required for nuclear transport. *Mol. Cell. Biol.* **17**, 3755-3767
13. Noguchi, E., Hayashi, N., Azuma, Y., Seki, T., Nakamura, M., Nakashima, N., Yanagida, M., He, X., Mueller, U., Sazer, S., and Nishimoto, T. (1996) Dis3, implicated in mitotic control, binds directly to Ran and enhances the GEF activity of RCC1. *EMBO J.* **15**, 5595-5605
14. Ohkura, H., Adachi, Y., Kinoshita, N., Niwa, O., Toda, T., and Yanagida, M. (1988) Cold-sensitive and caffeine supersensitive mutants of the *Schizosaccharomyces pombe dis* genes implicated in sister chromatid separation during mitosis. *EMBO J.* **7**, 1465-1473
15. Ohkura, H., Adachi, Y., Kinoshita, N., Niwa, O., Toda, T., and Yanagida, M. (1989) The fission yeast *dis2*$^+$ gene required for chromosome disjoining encodes one of two putative type 1 protein phosphatase. *Cell* **57**, 997-1007
16. Kinoshita, N., Goebl, M., and Yanagida, M. (1991) The fission yeast *dis3*$^+$ gene encodes a 110 kDa essential protein implicated in mitotic control. *Mol. Cell Biol.* **11**, 5839-5847
17. Mitchell, P., Petfalski, E., Shevchenko, A., Mann, M., and Tollervey, D. (1997) The exosome: a conserved eucaryotic RNA processing complex containing multiple 3′-5′ exoribonucleases. *Cell* **91**, 457-466
18. Hayashi, N., Yokoyama, N., Seki, T., Azuma, Y., Ohba, T., and Nishimoto, T. (1995) RanBP1, a Ras-like nuclear G-protein binding to Ran/TC4, inhibits RCC1 via Ran/TC4. *Mol. Gen. Genet.* **247**, 661-669
19. Nishiwaki, H., Hayashi, N., Irie, S., Chung, D.-H., Harashima, S., and Oshima, Y. (1987) Structure of the yeast *HIS5* gene responsive to general control of amino acid biosynthesis. *Mol. Gen. Gent.* **208**, 159-167
20. 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

21. Mitchell, D.A., Marshall, T.K., and Deschenes, R.J. (1993) Vectors for the inducible overexpression of glutathione S-transferase fusion proteins in yeast. *Yeast* **9**, 715-723
22. Noguchi, E., Hayashi, N., Nakashima, N., and Nishimoto, T. (1997) Yrb2p, Nup2p-related yeast protein has a functional overlap with Rna1p, yeast RanGAP protein. *Mol. Cell. Biol.* **17**, 2235-2246
23. 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 nuclear structure. *Proc. Natl. Acad. Sci. USA* **91**, 6880-6884
24. End, P., Gout, I., Fry, M.J., Panayotou, G., Dhand, R., Yonezawa, K., Kasuga, M., and Waterfield, M.D. (1993) A biosensor approach to probe the structure and function of the p85a subunit of the phosphatidylinositol 3-kinase complex. *J. Biol. Chem.* **268**, 10066-10075
25. Lim, J.-T., Kuroki, T., Ozaki, K., Kohsaki, H., Yamori, T., Tsuruo, T., Nakamori, S., Imaoka, S., Endo, M., and Nakamura, Y. (1997) Isolation of murine and human homologues of the fission-yeast *dis3*$^+$ gene encoding a mitotic-control protein and its overexpression in cancer cells with progressive phenotype. *Cancer Res.* **57**, 921-925
26. Ohtsubo, M., Ozaki, H., and Nishimoto, T. (1989) The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and bind to DNA. *J. Cell Biol.* **109**, 1389-1397
27. Frasch, M. (1991) The maternally expressed *Drosophila* gene encoding the chromatin-binding protein BJ1 is a homologue of the vertebrate gene regulator of chromosome condensation, RCC1. *EMBO J.* **10**, 1225-1236
28. Rad, M.R., Habbig, B., Jansen, G., Hattenhorst, U., Krol, M., and Hollenberg, C. (1997) Analysis of the DNA sequence of a 34,038 bp region on the left arm of yeast chromosome XV. *Yeast* **13**, 281-286
29. 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
30. 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
31. Hopper, A.K., Banks, F., and Evangelidis, V. (1978) A yeast mutant which accumulates precursor tRNAs. *Cell* **19**, 211-219
32. Forrester, W., Stutz, F., Rosbash, M., and Wickens, M. (1992) Defects in mRNA 3′-end formation, transcription initiation, and mRNA transport associated with the yeast mutation *prp20*: possible coupling of mRNA processing and chromatin structure. *Genes Dev.* **6**, 1914-1926
33. Sikorski, R.S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19-27