FEBS Letters 565 (2004) 106–110 FEBS 28329

Rrs1p, a ribosomal protein L11-binding protein, is required for nuclear export of the 60S pre-ribosomal subunit in *Saccharomyces cerevisiae*

Keita Miyoshi[a,b], Chiharu Shirai[a], Chihiro Horigome[a], Kazuhiko Takenami[b], Junko Kawasaki[b], Keiko Mizuta[a,b,*]

[a]*Department of Bioresource Science and Technology, Graduate School of Biosphere Science, Hiroshima University, Kagamiyama 1-4-4, Higashi-Hiroshima 739-8528, Japan*
[b]*Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Kagamiyama 1-3-1, Higashi-Hiroshima 739-8530, Japan*

Received 24 February 2004; accepted 18 March 2004

First published online 9 April 2004

Edited by Lev Kisselev

Abstract Rrs1p is a ribosomal protein L11-binding protein in *Saccharomyces cerevisiae*. We have obtained temperature-sensitive *rrs1* mutants by random PCR mutagenesis. [^{3}H]Methionine pulse-chase analysis reveals that the *rrs1* mutations cause a defect in maturation of 25S rRNA. Ribosomal protein L25-enhanced green fluorescent protein, a reporter of the 60S ribosomal subunit, concentrates in the nucleus with enrichment in the nucleolus when the *rrs1* mutants are shifted to the restrictive temperature. These results suggest that Rrs1p stays on the pre-60S particle from the early stage to very late stage of the large-subunit maturation and is required for export of 60S subunits from the nucleolus to the cytoplasm.

Keywords: Pre-rRNA processing; Nuclear export; Ribosome assembly; Ribosomal protein L11; *RRS1*

1. Introduction

Ribosome synthesis is a barometer of cell growth in yeast. Yeast cells produce about 2000 ribosomes/min when growing actively and are able to control ribosome synthesis in response to environmental changes [1]. Ribosomes are synthesized mainly in the nucleolus (reviewed in [2,3]). Ribosomal RNA (rRNA) is transcribed as a precursor RNA, and many ribosomal protein (RP) and non-RPs assemble with 35S rRNA, the largest detectable precursor, to form a large ribonucleoprotein particle. This particle corresponding to the 90S pre-ribosomal particle is converted to precursors of the 40S subunit and the 60S subunit by cleavage of the pre-rRNA and assembly of other factor proteins [4]. Several intermediates of the 60S subunit were identified by tandem affinity purification (TAP) followed by mass spectrometry [5–9]. The early pre-60 S ribosomal particle is produced in the nucleolus, and the later complex is transported to the nucleoplasm, and further to the cytoplasm. Export of the 60S subunit requires Crm1p/Xpo1p and Nmd3p, a shuttling NES-adaptor protein.

It has been demonstrated that more than 100 *trans*-acting factor proteins are involved in pre-rRNA processing and assembly of ribosomal subunits in the nucleolus. However, the biochemical functions of many of these proteins remain unclear. Rrs1p is one of the factors whose biochemical functions are unclear, although it is clearly essential for biogenesis of the 60S ribosomal subunit [10,11]. *RRS1* was originally isolated as the gene responsible for signal transduction for transcriptional repression of ribosomal genes due to a secretion block [10]. We clarified that Rrs1p has a role in the maturation of 25S rRNA and one in the assembly of the 60S ribosomal subunit [10,11]. Rrs1p has two-hybrid interactions with Ebp2p and Rpf2p, which are also essential for 60S subunit synthesis [12,13]. Both Rrs1p and Rpf2p can bind to Rpl11p, RP L11. Rpf2p is a member of the Imp4p superfamily that possesses the σ^{70}-like RNA-binding motif [6]. We proposed that Rrs1p and Rpf2p recruit Rpl11p to the pre-ribosomal particle [13]. Ebp2p was identified in pre-60S complexes co-purified from TAP-Ssf1p [5] and TAP-Nop7p [7], and Rfp2p was identified by TAP-Ssf1p [5]. However, Rrs1p has never been detected in the TAP-complexes as far as we know, even when they contain Ebp2p and/or Rpf2p.

Most of the factors of ribosome synthesis containing Rrs1p, Ebp2p, and Rpf2p are essential for cell growth and evolutionally conserved in eukaryotes. Homologues of many factor proteins were identified in proteomic analyses of nucleoli isolated from cultured human cells [14,15]. Moreover, it was suggested that *RRS1* was implicated in the underlying process occurring in the human Huntington's disease [16]. In order to obtain more details about the function of Rrs1p in yeast, we have isolated and characterized temperature-sensitive *rrs1* mutants. Here, we show that Rrs1p is required for the export of the 60S ribosomal subunit from the nucleus to the cytoplasm.

* Corresponding author. Fax: +81-82-424-7923.
E-mail address: kmizuta@hiroshima-u.ac.jp (K. Mizuta).

Abbreviations: TAP, tandem affinity purification; eGFP, enhanced green fluorescent protein; DsRed, red fluorescent protein; ORF, open reading frame; ts, temperature sensitive; cs, cold sensitive; HA, hemagglutinin; RP, ribosomal protein

0014-5793/$22.00
doi:10.1016/j.febslet.2004.03.087

2. Materials and methods

2.1. Random PCR mutagenesis and isolation of rrs1 mutants

Random mutagenesis of the *RRS1* gene was performed for 30 cycles, each consisting of 1 min at 91 °C, 1 min at 50 °C, and 3 min at 72 °C in the reaction mixture containing 1× PCR buffer (TaKaRa), 0.2 mM each of dNTPs, with the exception that concentration of dGTP or dATP was increased to 0.5 mM, 1.8 mM $MgCl_2$, 0.18 mM $MnCl_2$, 0.3 μM of each primer, pRS316 (*URA3*)-*RRS*1, and *Taq* DNA polymerase (TaKaRa). The PCR products were digested with *Xba*I and *Sna*BI at the 5′ and 3′ flanking sites of the *RRS1* open reading frame (ORF) and ligated with pRS313 (*HIS3*)-RRS1 at the same restriction sites. KM111 (W303a *rrs1*Δ::*LEU2* pRS316-*HA-RRS1*, [10]) was transformed with the library and replica-plated onto SC–His plate containing 5-fluoroorotic acid to isolate mutants (W303a *rrs1*Δ::*LEU2 pRS313-rrs1*). Colonies were picked up, stamped onto YPD plates, and incubated at 14, 18, 25, 35.5, or 37 °C. Each plasmid DNA recovered from candidates for temperature (ts)- or cold (cs)-sensitive mutant was reintroduced into KM111 [10] by plasmid shuffling. We constructed the strains in which *rrs1-84*, *rrs1-124*, *HA-rrs1-84*, and *HA-rrs1-124* alleles were integrated in the genomic DNA (W303a *rrs1*Δ::*HIS3 rrs1* integrated at *RRS1*).

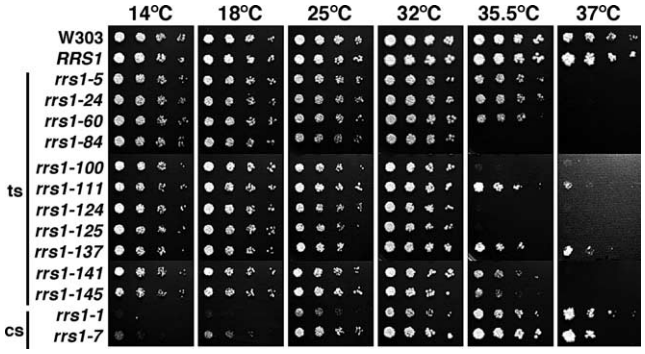

Fig. 1. Temperature-sensitive and cs phenotypes of the *rrs1* mutants. W303a and the *rrs1*Δ::*LEU2* strains containing *RRS1* or *rrs1* plasmids were cultured. Fivefold serial diluted cells were stamped onto YPD plates and incubated at the indicated temperatures. *rrs1-1* (KM112) was previously isolated [10].

2.2. Other methods

For Western blotting, anti-GFP antibodies (kindly provided by Silver; [18]), anti-HA antibodies (12CA5), and anti-PSTAIRE (sc-53) were used for detection of Rpl25p-enhanced green fluorescent protein (eGFP), hemagglutinin (HA)-Rrs1p, and Cdc28p, respectively. Horseradish peroxidase-conjugated donkey anti-rabbit IgG or sheep anti-mouse IgG was used as the secondary antibody. Signals were visualized by Enhanced Chemiluminescence (Amersham). Northern blotting, [*methyl*-^{3}H]methionine pulse-chase analysis and preparation of the ribosome fraction were performed as previously described [11,19].

3. Results and discussion

3.1. Isolation of rrs1 mutants

We obtained 11 ts and 1 cs mutants by using low fidelity PCR (Fig. 1). DNA sequencing revealed that each of the ts mutants has a mutated *rrs1* allele that causes one or several amino acid substitutions (Fig. 2). Most of the ts mutants have at least one amino acid substitution in two narrow regions, from 22 to 32 or from 61 to 66 amino acid residues. Several substitutions are shared by different alleles and some of them are conserved amino acid residues from yeast to human: *rrs1-84*, *-100*, and *-141* have D22G, and *rrs1-124* and *-125* have L61P. Others are conserved between budding yeast and human; *rrs1-100* and *-111* have N32D, and *rrs1-5* and *-60* have L65P (Fig. 2; [10]). Both *rrs1-5* and *rrs1-124* have only one amino acid substitution, indicating that both L65P and L61P confer a temperature sensitivity phenotype. One cs mutant, *rrs1-7*, has a nonsense mutation at codon 141. Compared with *rrs1-1*, the cs mutant that we previously isolated [10], *rrs1-7* is predicted to produce a larger protein than *rrs1-1*, and it shows less severe cold sensitivity (Figs. 1 and 2). This suggests that the cold sensitivity depends on the extent of the C-terminal truncation. The *rrs1-84*, *-124*, and *-125* mutants exhibit more

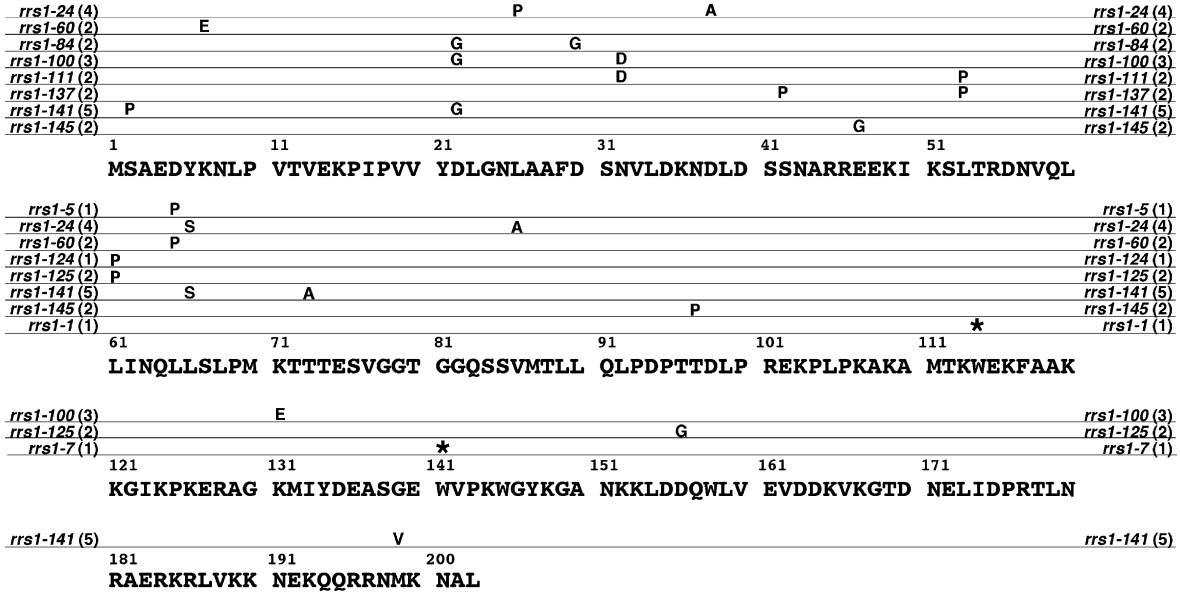

Fig. 2. Mutated alleles of *RRS1*. The complete Rrs1p amino acid sequence from 1 to 203 is shown in bold type. Amino acids substituted in each of the mutated alleles of *RRS1* are indicated in single-letter code above each altered amino acid. Stars indicate nonsense mutation. The number in parentheses indicates the number of substituted amino acids.

severe temperature sensitivity than the other mutants. As *rrs1-124* shares the amino acid substitution with *rrs1-125*, we performed further experiments using the *rrs1-84* and *rrs1-124* strains.

3.2. rrs1 alleles cause a defect in pre-rRNA processing

Expression levels of Rrs1p in *rrs1-84* and *rrs1-124* were determined by Western blot analysis using the HA-tagged version of the mutants. As shown in Fig. 3, approximately equal amount of Rrs1p was expressed in each strain at permissive temperature. At the restrictive temperature, *rrs1-124* expressed similar amount of Rrs1p compared to the wild-type cells, whereas in the *rrs1-84* cells, cellular concentration of Rrs1p appeared to be reduced, although the signal of HA-Rrs1p was still clearly detected.

We performed [*methyl*-^{3}H]methionine pulse-chase analysis to investigate whether the *rrs1* mutation caused a defect in pre-rRNA processing. At permissive temperature, the *rrs1-84* and *rrs1-124* mutants exhibited a slight retardation of the maturation of 25S rRNA compared with the wild-type cells (Fig. 4, lanes 1–12). When the cells were shifted to 37 °C, a severe effect was observed. In wild-type cells, most precursor rRNAs were processed to 25S and 18S after a 3-min chase (Fig. 4, lane 14). On the other hand, in the *rrs1* cells, the processing rate of the 35S pre-rRNA was slower than that of the wild-type cells at 37 °C. After a 10-min chase, a significant amount of the 27S pre-rRNA remained without further processing to 25S rRNA in the *rrs1* cells (Fig. 4, lanes 20 and 24), suggesting that the maturation of 25S rRNA is defective in the *rrs1-84* and *rrs1-124* cells at 37 °C. In contrast, most of the 20S pre-rRNA was processed to 18S rRNA in the *rrs1* cells after a 3-min chase at 37 °C (Fig. 4, lanes 18 and 22). The result indicates that the *rrs1* alleles cause a specific defect in the maturation of 25S rRNA, but not in the maturation of 18S rRNA.

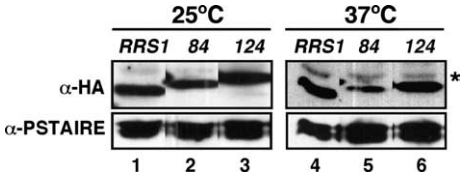

Fig. 3. Expression of Rrs1p in the *rrs1* mutants. The *HA-RRS1*, *HA-rrs1-84*, and *HA-rrs1-124* strains were grown at 25 °C and shifted to 37 °C for 4 h. Equal amounts (10 μg protein) of the cell extracts were used for Western blot analysis. Asterisk indicates non-specific bands.

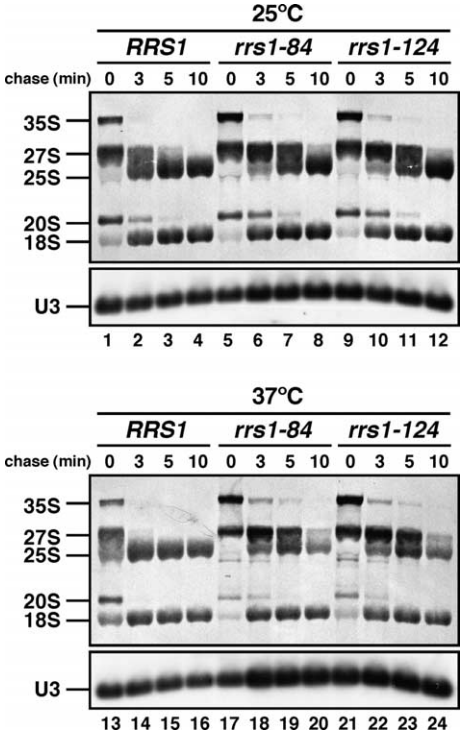

Fig. 4. The *rrs1* mutations cause a defect in pre-rRNA processing. The *RRS1*, *rrs1-84*, and *rrs1-124* cells were grown in SC–Met medium at 25 °C, and shifted to 37 °C for 4 h. Each culture was pulsed with [*methyl*-^{3}H]methionine (10 μCi/ml) for 3 min and chased. Samples were taken at the indicated times to prepare total RNA.

3.3. rrs1 mutations affect the transcriptional repression of both RP genes in response to a secretory defect

It was previously shown that a block in secretion caused transcriptional repression of both rRNA genes and RP genes [20,21]. *RRS1* was originally isolated as the wild-type allele of the cs *rrs1-1* mutation, which caused derepression of RP gene transcription when the secretory pathway was blocked [10]. We examined whether the *rrs1* mutations also had any effect on the transcriptional repression of RP genes and rRNA genes. Both *rrs1-84* and *rrs1-124* mutations attenuated the transcriptional repression of both RP and rRNA genes due to a secretory defect, which was caused by the addition of tunicamycin, an inhibitor of the secretory pathway at 33 °C, a semi-permissive temperature for the mutants (data not shown). Taken together, both the cs *rrs1-1* mutation [10] and the ts *rrs1-84* and *rrs1-124* mutations cause defects in both 60S subunit biogenesis and the secretory response, supporting our model in which the mechanism of transcriptional repression in response to a secretory defect is tightly linked to the normal regulatory mechanism that maintains ribosome synthesis [11].

3.4. rrs1 mutants are impaired in intranuclear transport and nuclear export of the 60S subunit

Before and during nuclear export, some alterations on the 60S ribosomal subunit could be required for release from the nucleolus and assembly into a competent export complex. We determined whether the *rrs1* mutants are impaired in the export of 60S ribosomal subunits from the nucleolus to the cytoplasm at the non-permissive temperature by visual assay for nuclear accumulation of Rpl25p-eGFP, which had been used as a reporter of the 60S ribosomal subunits (reviewed in [22]). At 25 °C, the signal of Rpl25p-eGFP was detected throughout the cells except the vacuoles in each strain. When the *rrs1* mutants were transferred to 37 °C, weak signal from the cytoplasm and dense signal from the nucleus were observed (Fig. 5A, 4 h). The signal from the cytoplasm was gradually disappeared and the signal from the nucleus was more concentrated with the passage of time (Fig. 5A, 6 and 8 h). Enlarged images showed that the dense signal of Rpl25p-eGFP was co-localized with that of Nop1p, a nucleolar protein, in the *rrs1 ts* mutants shifted for 6 h to 37 °C (Fig. 5B), suggesting that majority of Rpl25p-eGFP was accumulated into the nucleolus. Similar accumulation of Rpl25p-eGFP in the nucleus,

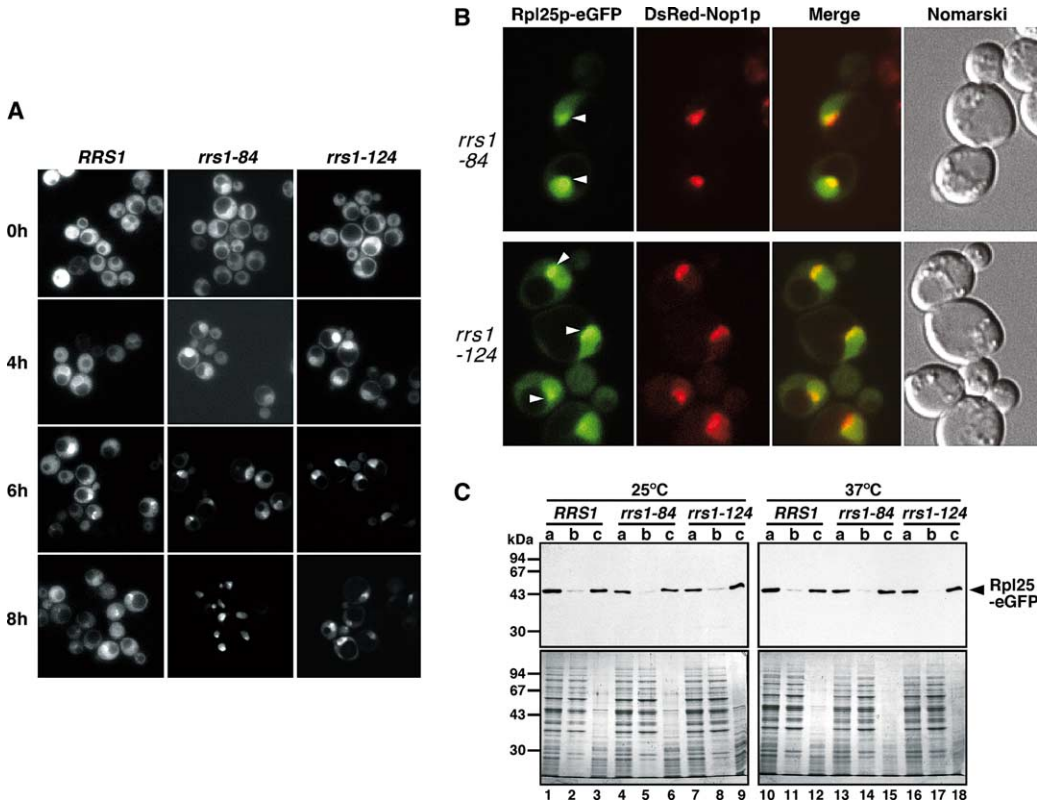

Fig. 5. *rrs1* mutants are defective in export of the 60S subunit from the nucleolus to the nucleoplasm and to the cytoplasm. Yeast strains *RRS1*, *rrs1-84*, and *rrs1-124* expressing both RPL25-eGFP and red fluorescent protein (DsRed)-NOP1 (plasmids were kindly provided by Gadal et al. [17]) were grown at 25 °C, and were shifted to 37 °C. (A) GFP signal was monitored at the indicated times after the temperature shift. (B) Six hours after the temperature shift, GFP and DsRed signals were observed. The magnification of the images is 5.06 times larger than that in (A). Arrows point to the nucleolus. (C) Before and 8 h after the temperature shift, cell cultures were treated with cycloheximide, lysed, and ultracentrifuged through low-salt sucrose cushions. Equivalent amounts of total fraction (a), supernatant (b), and ribosomal pellet (c) were subjected to SDS–PAGE and immunoblot analysis using anti-GFP antibodies (upper panel). Proteins were detected by Coomassie blue staining (lower panel).

with enrichment in the nucleolus, was observed in each of the *rrs1* ts mutants we isolated (data not shown). To ascertain that Rpl25p-eGFP associated with the ribosomal particle under the experimental conditions used, we analyzed extracts of the cells expressing Rpl25p-eGFP by ultracentrifugation and Western blotting. As shown in Fig. 5C, the majority of Rpl25p-eGFP was detected in the ribosomal pellet fraction even at the restrictive temperature for the *rrs1* mutants. These results suggest that Rrs1p is necessary for the intranuclear transport and nuclear export of the 60S ribosomal subunit.

The nuclear export of the 60S ribosomal subunit was previously shown to be mediated by the adaptor protein Nmd3p in a Crm1p/Xpo1p-dependent pathway [17,23]. It was suggested that the functions of Crm1p and Nmd3p in ribosomal subunit export are conserved from yeast to higher eukaryotes [24,25]. The early pre-60S particles are localized in the nucleolus, but later particles are released into the nucleoplasm. Rrs1p is localized both in the nucleolus and in the nucleoplasm [10], suggesting that it is a shuttling protein between the nucleolus and the nucleoplasm. In the absence of Rrs1p, the pre-60S particle may not be competent for nuclear export. We previously demonstrated that Rrs1p is required for the processing from 27SB to 25S rRNA, indicating that Rrs1p is assembled into the early 60S particles. In addition, we showed that *rrs1* ts mutations caused accumulation of Rpl25p-eGFP into the nucleus and then into the nucleolus. In order to deny that this is common to the mutants that lead to a defect in 60S synthesis, we investigated the localization of Rpl25p-eGFP in the *ebp2 ts* mutants. *EBP2* is required for 60S synthesis [12], and the *ebp2 ts* alleles caused a severe defect in both the maturation of 25S rRNA and assembly of 60S ribosomal subunit at the restrictive temperature (Horigome et al., unpublished data). However, in the *ebp2* cells, the signal of Rpl25p-eGFP was still dispersed in the cytoplasm with the weak accumulation in the nucleus even 8 h after the shift to the restrictive temperature (unpublished data). Therefore, we suggest that Rrs1p is specifically implicated in previous assembly steps, which need to be completed for nuclear export of the 60S ribosomal subunit. Taken together, the data suggest that Rrs1p stays on the pre-60S particle from the early stage to very late stage of the large-subunit maturation.

Rrs1p is an Rpl11p-binding protein. Rrs1p is highly conserved in eukaryotes and is 36.8% identical to its human homologue [10]. Several lines of evidence show the mammalian homologues of Rrs1p and Rpl11p to play important roles in various kinds of cellular responses in higher eukaryotes [16,26–28]. As Rrs1p was detected in the nucleolar fraction that was isolated from human cells [14,15], it is highly possible that human Rrs1p has a role in ribosome synthesis, like yeast Rrs1p. Further analysis of Rrs1p in yeast and the understanding of its function may shed light on the role of the human homologue in human diseases.

Acknowledgements: We thank E. Hurt and O. Gadal for plasmids, and P.A. Silver for antibodies. This research was supported by grants from MEXT, JSPS, and the Naito Foundation to K. Mizuta. K. Miyoshi is the recipient of a JSPS Pre-doctoral Research Fellowship.

References

[1] Warner, J.R. (1999) Trends Biochem. Sci. 24, 437–440.
[2] Venema, J. and Tollervey, D. (1999) Annu. Rev. Gen. 33, 261–311.
[3] Kressler, D., Linder, P. and de la Cruz, J. (1999) Mol. Cell. Biol. 19, 7897–7912.
[4] Dragon, F., Gallagher, J.E.G., Compagnone-Post, P.A., Mitchell, B.M., Porwancher, K.A., Wehner, K.A., Wormsley, S., Settlage, R.E., Shabanowitz, J., Osheim, Y., Beyer, A.L., Hunt, D.F. and Baserga, S.J. (2002) Nature 417, 967–970.
[5] Fatica, A., Cronshaw, A.D., Dlakic, M. and Tollervey, D. (2002) Mol. Cell 9, 341–351.
[6] Wehner, K.A. and Baserga, S.J. (2002) Mol. Cell 9, 329–339.
[7] Baßler, J., Grandi, P., Gadal, O., Leßmann, T., Petfalski, E., Tollervey, D., Lechner, J. and Hurt, E. (2001) Mol. Cell 8, 517–529.
[8] Nissan, T.A., Baßler, J., Petfalski, E., Tollervey, D. and Hurt, E. (2002) EMBO J. 21, 5539–5547.
[9] Harnpicharnchai, P., Jakovljevic, J., Horsey, E., Miles, T., Roman, J., Rout, M., Meagher, D., Imai, B., Guo, Y., Brame, C.J., Shabanowitz, J., Hunt, D.F. and Woolford Jr., J.L. (2001) Mol. Cell 8, 505–515.
[10] Tsuno, A., Miyoshi, K., Tsujii, R., Miyakawa, T. and Mizuta, K. (2000) Mol. Cell. Biol. 20, 2066–2074.
[11] Miyoshi, K., Tsujii, R., Yoshida, H., Maki, Y., Wada, A., Matsui, Y., Toh-e, A. and Mizuta, K. (2002) J. Biol. Chem. 277, 18334–18339.
[12] Tsujii, R., Miyoshi, K., Tsuno, A., Matsui, Y., Toh-e, A., Miyakawa, T. and Mizuta, K. (2000) Genes Cells 5, 543–553.
[13] Morita, D., Miyoshi, K., Matsui, Y., Toh-e, A., Shinkawa, H., Miyakawa, T. and Mizuta, K. (2002) J. Biol. Chem. 277, 28780–28786.
[14] Andersen, J.S., Lyon, C.E., Fox, A.H., Leung, A.K.L., Lam, Y.W., Steen, H., Mann, M. and Lamond, A.I. (2002) Curr. Biol. 12, 1–11.
[15] Scherl, A., Couté, Y., Déon, C., Callé, A., Kindbeiter, K., Sanchez, J.-C., Greco, A., Hochstrasser, D. and Diaz, J.-J. (2002) Mol. Biol. Cell 13, 4100–4109.
[16] Fossale, E., Wheeler, V.C., Vrbenac, V., Lebel, L.-A., Teed, A., Mysore, J.S., Gusella, J.F., MacDonald, M.E. and Persichetti, F. (2002) Hum. Mol. Genet. 11, 2233–2241.
[17] Gadal, O., Strauß, D., Kessl, J., Trumpower, B., Tollervey, D. and Hurt, E. (2001) Mol. Cell. Biol. 21, 3405–3415.
[18] Seedorf, M., Damelin, M., Kahana, J., Taura, T. and Silver, P.A. (1999) Mol. Cell. Biol. 19, 1547–1557.
[19] Miyoshi, K., Miyakawa, T. and Mizuta, K. (2001) Nucleic Acids Res. 29, 3297–3303.
[20] Mizuta, K. and Warner, J.R. (1994) Mol. Cell. Biol. 14, 2493–2502.
[21] Li, B. and Warner, J.R. (1996) J. Biol. Chem. 271, 16813–16819.
[22] Tschochner, H. and Hurt, E. (2003) Trends Cell Biol. 13, 255–263.
[23] Ho, J.H.-N., Kallstrom, G. and Johnson, A.W. (2000) J. Cell Biol. 151, 1057–1066.
[24] Trotta, C.R., Lund, E., Kahan, L., Johnson, A.W. and Dahlberg, J.E. (2003) EMBO J. 22, 2841–2851.
[25] Thomas, F. and Kutay, U. (2003) J. Cell Sci. 116, 2409–2419.
[26] Lohrum, M.A.E., Ludwig, R.L., Kubbutat, M.H.G., Hanlon, M. and Vousden, K.H. (2003) Cancer Cell 3, 577–587.
[27] Ishizuka, A., Siomi, M.C. and Siomi, H. (2002) Genes Dev. 16, 2497–2508.
[28] Geiss, G.K., An, M.C., Bumgarner, R.E., Hammersmark, E., Cunningham, D. and Katze, M.G. (2001) J. Virol. 75, 4321–4331.