Rpf2p, an Evolutionarily Conserved Protein, Interacts with Ribosomal Protein L11 and Is Essential for the Processing of 27 SB Pre-rRNA to 25 S rRNA and the 60 S Ribosomal Subunit Assembly in Saccharomyces cerevisiae*

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Saccharomyces cerevisiae Rrs1p is a nuclear protein that is essential for the maturation of 25 S rRNA and the 60 S ribosomal subunit assembly. In two-hybrid screening, using RRS1 as bait, we have cloned YKR081c/RPF2. Rpf2p is essential for growth and is mainly localized in the nucleolus. The amino acid sequence of Rpf2p is highly conserved in eukaryotes from yeast to human. Similar to Rrs1p, Rpf2p shows physical interaction with ribosomal protein L11 and appears to associate with preribosomal subunits fairly tightly. Northern, methionine-pulse-chase, and sucrose density gradient ultracentrifugation analyses reveal that the depletion of Rpf2p results in a delayed processing of pre-rRNA, a decrease of mature 25 S rRNA, and a shortage of 60 S subunits. An analysis of processing intermediates by primer extension shows that the Rpf2p depletion leads to an accumulation of 27 SB pre-rRNA, suggesting that Rpf2p is required for the processing of 27 SB into 25 S rRNA.

**Eukaryotic ribosome, composed of four rRNAs (25–28, 18, 5.8, and 5 S) and about 80 ribosomal proteins, is synthesized through many processes (1, 2). Ribosomal proteins newly synthesized in cytoplasm are transported into the nucleolus, which is the site of the transcription of rRNA genes, the modification and processing of pre-rRNAs, and the assembly of ribosomal subunits. In Saccharomyces cerevisiae, a 9.1-kb rDNA unit encoding all four rRNAs is tandem repeated 100–200 times on chromosome XII. Three of the four rRNAs (25, 18, and 5.8 S) are transcribed as a single pre-rRNA by RNA polymerase I. The 35 S pre-rRNA, detected as the largest pre-rRNA, is cleaved at two internal transcribed spacer (ITS)\(^1\) sequences and two external transcribed spacer (ETS) sequences (Fig. 1). During pre-rRNA processing, ribosomal proteins that are transported into the nucleolus assemble onto pre-rRNAs to yield preribosomal particles. Thus, the processes of rRNA processing and subunit assembly are closely linked to each other. It has been reported (3, 4) that as many as 100 proteins are implicated in the necessary steps of these processes, but the regulatory mechanism and clear function of each factor remain to be elucidated.

In yeast cells, ribosome synthesis is strictly regulated mainly at the level of transcription according to the changes in environmental conditions such as carbon source upshift (5, 6), mild heat shock (7, 8), amino acid starvation (9), nitrogen limitation (10), and a secretory defect (12, 13). RRS1 was originally isolated as the wild type allele complementing the rrs1–1 mutation in which the transcription of ribosomal protein genes was derepressed when the secretory pathway was blocked (14). Rrs1p is an essential nuclear protein of 203 amino acids with an important function in the maturation of 25 S rRNA and the 60 S ribosomal subunit assembly. However, it is unknown how Rrs1p functions in ribosome biogenesis. To learn the function of Rrs1p, we performed yeast two-hybrid screening by using RRS1 as bait. In the screening, we had previously isolated EBP2 encoding the yeast homolog of human EBNA1-binding protein 2 and demonstrated that Ebp2p had an essential role in ribosome biogenesis (15).

Here, we isolate YKR081c/RPF2 in two-hybrid screening by using RRS1 as bait. Independently, it has very recently been demonstrated (16) that Rpf2p is involved in a discrete precursor to the 60 S ribosomal subunit, which is copurified with Ssf1p. Both Ssf1p and Rpf2p are members of the Imp4 superfamily that possess the \(\sigma^{70}\)-like motif, an eukaryotic RNA binding domain with prokaryotic origins (17). The amino acid sequence of Rpf2p is highly conserved in eukaryotes from yeast to human. We show that Rpf2p interacts with the ribosomal protein L11 (Rpl11p) and is required for proper ribosome biogenesis in cooperation with Rrs1p. We discuss the role of Rpf2p in the regulation of ribosome synthesis through interaction with Rrs1p.

MATERIALS AND METHODS

Yeast Strains and Media—The yeast strains used are listed in Table I. Yeast cells were grown in YPD-rich medium, synthetic complete medium containing 2% glucose (SC) or 2% galactose (SCGal), or SC dropout medium depending on the plasmid markers (18). Yeast transformation was performed by a lithium acetate procedure (19).

Plasmid Construction—A plasmid containing GAL1 promoter-controlled RPF2 was constructed as follows. The HpaI-EcoRI fragment containing the GAL1 promoter from the pNV7 vector (20) was inserted into a single copy plasmid, pRS414 (21). The NruI-XhoI PCR fragment containing the RPF2 open reading frame (ORF) and the terminator (415 bp) produced with a set of primers (5′-TTTTCCGGAATGATTAGAC-
CGTAAAAACCAAG-3' and 5'-TTTCTCGAGTACTCTGAGATCTGTTAGAAGAATGG-3') was inserted just after the GAL1 promoter of the resulting plasmid. A plasmid expressing Myc-tagged Rpf2p was constructed by the insertion of the HindIII-PstI PCR fragment containing the upstream promoter region of RPF2 (1,118 bp) and the RPF2 ORF produced with a set of primers (5'-TTTAAAGCTTACGGATCCATGG-3' and 5'-TTTCTCGAGTACTCTGAGATCTGTTAGAAGAATGG-3') into a single copy vector CTF, YCplac22 containing 9× Myc epitope and the TDH2 terminator, kindly provided by D. Kornitzer. A plasmid expressing hemagglutinin (HA)-tagged Rpl11p was constructed by inserting the BamHI-SmaI PCR fragment of the RPL11A ORF produced with a set of primers (5'-GGGAATTCATGGATCCATGTCTGCCAAAGC-3' and 5'-GGGAATTCATGGATCCATGTCTGCCAAAGC-3') into pRS116-GAL-HA-BS, kindly provided by K. Tanaka. A plasmid containing rDNA for a DNA sequencing ladder were produced with a set of primers (5'-H11032 and 5'-H11032).

**Indirect Immunofluorescence**—Indirect immunofluorescence microscopy was done as described previously (15, 23). Anti-Myc antibody (Berkeley Antibody), anti-HA antibody (Berkeley Antibody), and anti-GFP antibody (kindly provided by P. A. Silver; Ref. 24) were diluted 1:1,000, 1:1,000, and 1:5,000, respectively. Secondary antibodies (rho-
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Fig. 2. Rpf2p shows physical interaction with Rrs1p and Rpl11p. A, yeast two-hybrid interaction. Plasmids expressing the Rrs1p and Ebp2p fusion proteins were used as a positive control. BD, DNA-binding domain; AD, activation domain. B, co-immunoprecipitation of Rpf2p-Myc with HA-Rpl11p. Extracts of strains KM406 (RPL11A-HA RPF2-myc, lanes 1, 3, 5, and 7), KM404 (RPF2-myc, lanes 2 and 4), and KM407 (HA-RPL11A, lanes 6 and 8) were immunoprecipitated with anti-HA or anti-Myc monoclonal antibodies. The immunoprecipitates (corresponding to 6.0 μl of cell extract) and the extracts prior to precipitation (input; corresponding to 0.1 μl of cell extract) were analyzed by SDS-PAGE and immunoblotting.

Fig. 3. Rpf2p-Myc is localized in the nucleus with enrichment in the nucleolus. A, Western blotting of Rpf2p-Myc. Cell extracts from W303a (RPF2, lane 1) and KM404 (RPF2-myc, lane 2) were subjected to SDS-PAGE and Western blotting using anti-Myc antibody (α-myc). The positions of size markers are shown on the left. B, intracellular localization of Rpf2p-Myc detected by indirect immunofluorescence. KM404 (RPF2-myc NOP1-GFP) strain cells were grown in SC medium at 28°C to early log phase. Cells were stained with anti-Myc or anti-GFP (α-GFP) antibodies and 4',6'-diamidino-2-phenylindole (DAPI). The morphology of the cells was observed by differential interference contrast (DIC). Arrowheads indicate the boundary between the chromatin region and the nucleolus.

Fig. 4. Rpf2p-Myc associates with the ribosomal particle. A, panel a, yeast KM404 cells were treated with cycloheximide, lysed, and centrifuged through low salt sucrose cushions. Equivalent amounts of cell extract (total, lane 1), supernatant (sup, lane 2), and ribosome pellets (ppt, lane 3) were subjected to SDS-PAGE and immunoblot analysis using anti-Myc antibodies and anti-Rp3p antibodies (kindly provided by J. R. Warner). Panel b, ribosome pellets were treated with indicated concentrations of LiCl and centrifuged again through sucrose cushions containing the same concentrations of LiCl. Ribosome pellets were subjected to SDS-PAGE and immunoblot analysis. B, Rpf2p-myc cosediments with free 60 S ribosomal subunits. The polysome profile of ribosomes isolated from KM404 cells and separated using a 7–47% sucrose gradient is shown. Fractions from the gradient shown were collected, and proteins were precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE and immunoblotting. Rpl3p was used as a marker of 60 S ribosomal subunits.

RESULTS

Rpf2p Interacts with Rrs1p—Rrs1p, an evolutionarily conserved nuclear protein, is required for pre-rRNA processing and the proper assembly of ribosomal subunits in S. cerevisiae (14). To identify proteins that physically interact with Rrs1p, we performed yeast two-hybrid screening on the yeast cDNA library using RRS1 as bait. Among the 21 cDNA clones isolated in this screen, 3 cDNA clones had most of the YKR081c-RPP2 sequence. In a yeast two-hybrid assay, the HIS3 reporter gene was activated in the presence of a set of the Gal4p activation domain-Rpf2p fusion protein and the lexA binding domain.
Rrs1p fusion protein and in the presence of a replaced set (Fig. 2A, lanes 2 and 4). Another reporter gene, lacZ, was also activated in the same yeast transformant cells (data not shown).

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localized to the nucleus with enrichment in the nucleolus, similar to Rrs1p (14). The amino acid sequence of Rpf2p is encoded a protein consisting of 344 amino acids with an isoelectric point of 9.75. The amino acid sequences are also found in the genome sequences of plants (Fig. 4B). Another reporter gene, Rpf2p-Myc, was detected in a ribosomal pellet fraction. Cell extract containing Rpf2p-Myc was analyzed by indirect immunofluorescence microscopy. Nop1p-GFP, used as a nucleolar protein, was detected in the nucleoplasm. This suggests that Rpf2p is localized to the nucleus with enrichment in the nucleolus, similar to Rrs1p (14).

An RPF2-null allele was created by replacing one of the RPF2 ORFs of W303 with the HIS3 gene. Southern blot analysis of chromosomal DNA isolated from a candidate of W303 rpf2Δ/+ demonstrated that the resultant diploid cells carried one intact RPF2 gene and one disrupted by the insertion of HIS3 (data not shown). Twenty tetrads from the transformant were dissected. All tetrads yielded only two viable spores, and all of them were His−, indicating that RPF2 is essential (data not shown).

To learn the subcellular localization of Rpf2p, we constructed a plasmid to express Rpf2p tagged with nine repeats of the Myc epitope at the C terminus. The single copy plasmid containing its own promoter-regulated RPF2-myc could suppress the lethality of the rpf2 null mutation, indicating that the construct is biologically functional. Western blotting with anti-Myc antibodies detected Rpf2p-Myc as a band with an apparent molecular mass of 67 kDa (Fig. 3A), which is somewhat larger than the predicted molecular weight of the protein with nine repeats of the Myc tag (50 kDa). The subcellular localization of Rpf2p-Myc was analyzed by indirect immunofluorescence microscopy. Nop1p-GFP, used as a nucleolar protein, was detected in the region adjacent to the 4.6-diamidino-2-phenylindole-stained region, the nucleolus (Fig. 3B). Rpf2p-Myc was detected mainly in the same region as the Nop1p-GFP and also in the nucleoplasm. This suggests that Rpf2p is localized to the nucleus with enrichment in the nucleolus, similar to Rrs1p (14).

Rpf2p Interacts with Rpl11p and Associates with the 60 S Preribosomal Subunit—Because Rrs1p showed physical interaction with both Ebp2 (15) and Rpl11p (Ref. 25; for nomenclature, see Ref. 26), we checked whether Rpf2p interacts with Ebp2p and Rpl11p. Rpf2p showed two-hybrid interaction with Rpl11p but not with Ebp2p (Fig. 2A, lanes 5–7). The results were reproducible, and the assay using the lacZ reporter gene showed similar results (data not shown). Immunoprecipitation analysis also revealed that Rpf2p-Myc interacts with HA-Rpl11p (Fig. 2B).

We next examined whether Rpf2p associates with ribosomal particles. Cell extract containing Rpf2p-Myc was analyzed by ultracentrifugation and Western blotting. As shown in Fig. 4A, panel a, Rpf2p-Myc was detected in a ribosomal pellet fraction.
To investigate how tightly Rpf2p associates with the ribosomal particle, the ribosome pellets were treated with various concentration of LiCl and subjected to a second ultracentrifugation. The pattern of Western blotting shows that the effect of LiCl on the dissociation of Rpf2p-Myc was very similar to that of ribosomal protein L3 (Rpl3p), which was used as the large subunit reporter. A part of Rpf2p remains to associate with the ribosomal particle even in the presence of 1M LiCl (Fig. 4A, panel b).

To determine the type of ribosome with which Rpf2p associates, we performed sucrose density gradient ultracentrifugation followed by the detection of Rpf2p by Western blotting. The analysis revealed that Rpf2p-Myc was involved in fractions containing free 60 S ribosomal subunits (Fig. 4B). Considering that Rpf2p-Myc is localized in the nucleus, the results suggest that Rpf2p tightly associates with 60 S preribosomal subunits and/or 66 S preribosomal subunits. We demonstrated quite similar results previously with HA-Rrs1p, suggesting that Rrs1p associates fairly tightly with 66 S and/or 60 S preribosomal subunits (25).

Depletion of Rpf2p Results in an Abnormal Polysome Profile—Because Rpf2p was localized mainly in the nucleolus and associated with the ribosomal particle, Rpf2p was expected to have a role in ribosome biogenesis. To test this expectation, we analyzed a polysome pattern from the Rpf2p-depleted cells on sucrose density gradients. To achieve conditional expression of RPF2, we constructed a strain in which the chromosomal RPF2 gene was disrupted, and a plasmid containing the GAL1-promoter driven RPF2 gene was introduced. This strain, KM403, was shifted from galactose medium to glucose medium to deplete Rpf2p. After 8 h in YPD medium, the growth of the cells slowed gradually and was severely impeded after 16 h (Fig. 5A). We performed sucrose density gradient ultracentrifugation using the cells after 8 h in glucose. Lower amounts of both 80 S monosomes and polysomes were detected in the profile from the Rpf2p-depleted cells, even though cell lysates with equal A260 units were analyzed (Fig. 5B). Furthermore, the polysome profile showed that the depletion of Rpf2p caused an accumulation of the 40 S ribosomal subunits, and the appearance of half-mer polysomes, which contain 43 S initiation complexes, stalled at the AUG start codon (Fig. 5B; Ref. 27). These results indicate that Rpf2p depletion affects 60 S ribosomal subunit assembly.

Pre-rRNA Is Processed Slowly and Is Unstable in the Rpf2p-depleted Cells—To investigate pre-rRNA synthesis and its processing in Rpf2p-depleted cells, we performed [methyl-3H]methionine pulse-chase analysis as newly synthesized pre-rRNA was immediately methylated (28, 29). As shown in Fig. 1,
the 35 S pre-rRNA, the longest detectable precursor, is cleaved to the 27 and the 20 S pre-rRNAs, which are further processed to the mature 25 and 18 S rRNAs, respectively. In wild type cells, most precursor rRNAs were processed to 25 and 18 S after a 3-min chase (Fig. 6A, lane 2). In the GAL1-RPF2 cells that were transferred into a glucose medium for 8 h, the processing rate of the 35 S pre-rRNA was slower than that of wild type cells, and a significantly smaller amount of the mature 25 S rRNA was produced. After a 3-min chase, much of the 27 S pre-rRNA remained in Rpf2p-depleted cells without further processing to 25 S rRNA (Fig. 6A, lane 7). Furthermore, the amount of produced 25 S rRNA appeared to be less than that expected from the total amount of precursors detected at the beginning of the chase (Fig. 6A, lanes 6–10), suggesting that the intermediates are unstable in Rpf2p-depleted cells. In contrast, 18 S rRNA appeared to be rather stable for at least 30 min, although a smaller amount of 18 S was detected in the Rpf2p-depleted cells than in wild type cells. Small nucleolar RNA U3 was used as a loading marker.

We performed Northern analysis to show the steady-state levels of the mature rRNAs in wild type and Rpf2p-depleted cells after the shift to a glucose medium for various periods of time. Following transfer of the GAL1-RPF2 strain to glucose medium, the steady-state levels of both 25 and 18 S rRNAs began to decrease significantly, 40 S subunits accumulate, and half-mer polysomes appear, suggesting a shortage of mature 60 S ribosomes. It was suggested that Rrb1p, a yeast homolog of the smallest subunit of chromatin assembly factor 1 (CAF1), forms a direct complex with Rpl3p and assists its functions.

DISCUSSION

In this paper we have isolated the RPF2 gene as an Rrs1p-interacting factor and showed that RPF2 is an essential gene for ribosome biogenesis. [methyl-3H]methylionine pulse-chase analysis has revealed that the depletion of Rpf2p results in retarded pre-rRNA processing and the inhibition of 25 S rRNA production. Consistent with this, the polysome profiles show that, in Rpf2p-depleted cells, 80 S monosomes and polysomes decrease significantly, 40 S subunits accumulate, and half-monomer polysomes appear, suggesting a shortage of mature 60 S ribosomal subunits. Northern analysis for steady-state levels of rRNA shows that 25 S rRNA is severely reduced in Rpf2p-depleted cells. A comparison of the timing of the decrease of these two sets of mature rRNAs suggests that the decrease of 18S rRNA may be a secondary effect caused by the inhibition of 25 S rRNA production. In total, the results indicate that Rpf2p has important roles in the maturation of 25 S rRNA and the assembly of 60 S ribosomal subunits. Primer extension analysis has revealed that both Rpf2p and Rrs1p are required for the processing of 27 SB/27 SB1 intermediates to the mature 25 S rRNA. In the pre-rRNA processing pathway (Fig. 1), 27 SA2 is processed via two alternative pathways. The major pathway involves cleavage at site A0, generating the 27 SA3 intermediate. This intermediate is very rapidly digested to yield 27 SB3. In the minor pathway, about 15% of the 27 SA3 molecules are processed at site B1L, generating 27 SB1. The subsequent processing pathways of both 27 SB3 and 27 SB1 appear to be identical. The processing at sites C1 and C2 generates 7 S pre-rRNA and the mature 25 S rRNA. Rpf2p and Rrs1p are therefore essential for ITS2 processing. Recent works (Refs. 17, 30–32, and reviewed in Ref. 33) using a tandem affinity purification tag and mass spectrometry have discriminated several intermediates in a multistep pathway of 60 S preribosome maturation. Rpf2p was detected in one intermediate that was isolated by copurification with Ssf1p (17). The intermediate appears to contain 27 SB pre-rRNA, consistent with our conclusion.

Pre-rRNA processing and the assembly of ribosomal subunits are tightly linked to each other because many ribosomal proteins associate with the 35 S pre-rRNA at early steps prior to its cleavage. About 100 protein trans-acting factors have been found to be involved in both pre-rRNA processing and ribosomal subunit assembly (3, 4). Some proteins are considered to have enzymatic activities such as endo-RNase, exo-RNase, 2'-O-ribose methyltransferase, and rRNA pseudouridine synthase. A large class of the trans-acting factors contains putative RNA helicases that may be required to melt structures of pre-rRNA. Some proteins might have a role in maintaining the structure of the complex containing pre-rRNA(s) and many ribosomal proteins. The depletion of such a factor may cause a structural change in the complex and prevent further processing of pre-rRNA. It is highly possible that pre-rRNAs that are not processed normally are degraded rapidly. In the [methyl-3H)methylionine pulse-chase analysis, the depletion of Rpf2p results in a decrease of 25 S production by the slow processing of 35 S pre-rRNA and the degradation of the intermediates. This suggests that Rpf2p has a role in maintaining the normal structure of preribosomal particles.

The consequences of Rpf2p depletion are quite similar to those observed in Rrs1p-depleted cells; the depletion of Rrs1p causes a slow maturation of 25 S rRNA, an abnormal polysome profile, and a less severe decline of 18 S rRNA levels compared with those of 25 S rRNA (14). Both Rrs1p and Rpf2p are highly conserved proteins throughout evolution. It is highly possible that the regulatory system for ribosome synthesis containing Rpf2p and Rrs1p is conserved in eukaryotes from yeast to human. It is interesting to define their shared and distinctive functions.

We have shown that Rpf2p interacts with Rpl11p and is detected in the ribosomal fraction containing free 60 S ribosomal subunits. Rpl11p is localized near the top surface of the central protuberance of the 60 S subunit (34, 35). It is suggested that Rpl11p interacts with Rps13p to form a bridge with the 40 S subunit, the 5 S rRNA binding protein Rpl5p, and the elbow of the P site-bound tRNA (36). This is consistent with the evidence showing that Rpf2p has a role in the processing of 27 S pre-rRNA to 25 S rRNA, because Rpl11p is considered to be a late-associating 60 S ribosomal protein (reviewed in Ref. 4). Recent studies have revealed that several factors are required for the correct assembly of a specific ribosomal protein into preribosomal particles. It was suggested that Rrb1p, a yeast homolog of the smallest subunit of chromatin assembly factor 1 (CAF1), forms a direct complex with Rpl3p and assists its interaction with the rRNA precursor as a chaperone (37), and
that Rrp7p is required for the correct assembly of Rps27A/Bp into pre-40 S ribosomal particles (38). Sqt1p, a protein containing multiple WD repeats, is involved in the assembly of Rpl10p/Qsr1p on the 60 S ribosomal protein (39). It is possible that every ribosomal protein needs a specific chaperone for its assembly to preribosomal particles. Fig. 8 presents our model for the function of Rpf2p in Rpl11p recruitment to pre-rRNA. Rpf2p has a RNA-binding domain (17), but it has not yet been determined whether Rps1p itself has the ability to bind to RNA. It is possible that Rpf2p is required for the binding of Rps1p to pre-rRNA. The fairly tight association of Rpf2p with the preribosomal particle suggests that this factor may have another role in addition to the recruitment of ribosomal protein onto pre-rRNA. Further studies about the protein trans-acting factors are needed to clearly understand how ribosomal subunits are assembled.

Acknowledgments—We thank J. R. Warner for the anti-Rpl3p antibody, P. A. Silver for the anti-GFP antibody, R. Sternglanz for a yeast strain and plasmids, and Y. Kikuchi, Y. Nogi, K. Tanaka, and D. Kornitzer for plasmids.

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