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ESF1 is required for 18S rRNA synthesis in *Saccharomyces cerevisiae*

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ABSTRACT

We report that Esf1p (Ydr365cp), an essential, evolutionarily conserved nucleolar protein, is required for the biogenesis of 18S rRNA in *Saccharomyces cerevisiae*. Depletion of Esf1p resulted in delayed processing of 35S precursor and a striking loss of 18S rRNA. Esf1p physically associated with ribosomal proteins and proteins involved in 18S rRNA biogenesis. Consistent with its role in 18S rRNA biogenesis, Esf1p also physically associated with U3 and U14 snoRNAs, but did not appear to be a core component of the SSU processome. These data indicate that Esf1p plays a direct role in early pre-rRNA processing.

INTRODUCTION

Ribosome biosynthesis is one of the major metabolic activities in cells, and is highly conserved from yeast to humans. It occurs mainly in the nucleolus, a specialized compartment in the nucleus. Current knowledge of ribosome biogenesis is largely derived from studies of the yeast *Saccharomyces cerevisiae* (1,2). This process starts with the transcription of ribosomal RNA (rRNA) genes by RNA polymerase I and III into 35S pre-rRNA and 5S pre-rRNA. The 35S precursor is extensively modified and cleaved into the mature 18S, 5.8S and 25S rRNA through the coordinated action of a variety of endonucleases, exonucleases, RNA helicases and other protein factors (Fig. 1).

The U3 small nucleolar RNA (snoRNA), together with associated proteins, plays a central role in the 18S rRNA biogenesis (1,2) and is essential for the early cleavage events at sites A_0, A_1 and A_2 (1–3). Recently, protein complexes that contain the U3 snoRNA and participate in the biogenesis of 40S ribosome subunits have been identified. Dragon and colleagues identified a large nucleolar ribonucleoprotein (RNP), called the small subunit (SSU) processome, required for 18S ribosomal RNA biogenesis (4). It contains the U3 snoRNA and at least 28 proteins, including 17 polypeptides (Utp1–17, for 'U three protein') that had not previously been shown to be associated with U3 or implicated in pre-rRNA processing events. We have recently discovered that the SSU processome consists of at least three distinct 'sub-complexes' and each physically associates with U3 snoRNA (5). Grandi and colleagues reported 90S pre-ribosomes formed at very early stages of ribosome biogenesis (6). The purified 90S pre-ribosomes contained not only the U3-specific proteins, but also other nucleolar proteins with a known role in 18S rRNA processing and 40S subunit assembly and additional uncharacterized proteins. However, seven components identified in the SSU processome (Snu13p, Utp3p, Utp5p, Utp7p, Utp11p, Utp14p and Utp16p) were not found in the isolated 90S pre-ribosomes.

In addition to the U3 snoRNA, snoRNAs U14, snR10 and snR30 are required for early cleavages at sites A_1 and A_2 and hence also contribute to 18S rRNA biogenesis (7–9). However, the protein complexes containing these RNAs are less well-characterized than the U3-containing SSU processome. Furthermore, despite the application of several proteomic approaches to characterize early pre-ribosomal protein complexes (4–6) it is likely that not all proteins involved have been detected (4).

We recently carried out a large-scale survey of non-coding RNA processing phenotypes in yeast mutants using a microarray-based approach (10). A mutated version of *YDR365c*, a gene that is co-regulated with established rRNA processing factors at the transcriptional level (11), displayed an array phenotype suggesting involvement in ribosome biogenesis (10). Ydr365cp has not been identified in the protein complexes described above, although it has been associated with different rRNA processing factors in other large-scale studies (12,13). Here, we provide genetic and biochemical evidence that *YDR365c* is required for the early cleavages at sites A_0, A_1 and A_2 that lead to 18S rRNA synthesis, although it does not appear to be a core component of the SSU processome. Owing to its apparently direct role in 18S rRNA

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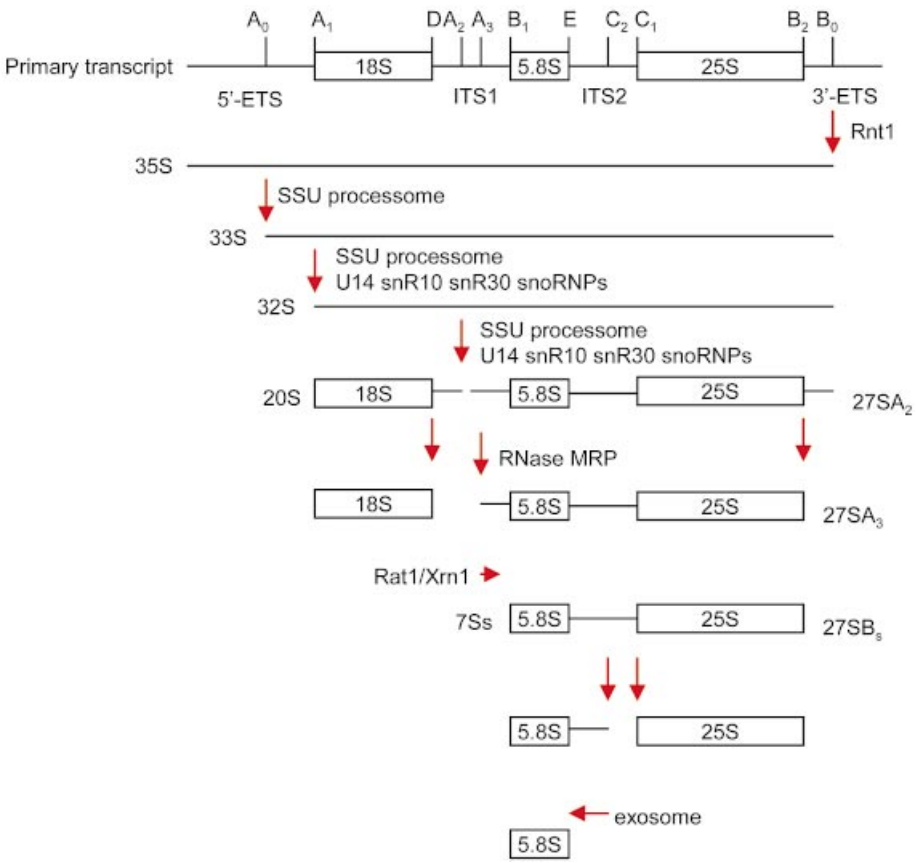

Figure 1. The major pre-rRNA processing pathway in *S.cerevisiae*. The primary transcript contains the sequences of mature 18S, 5.8S and 25S rRNA separated by two internal transcribed spacers (ITS1 and ITS2) and flanked by two external transcribed spacers (5′-ETS and 3′-ETS). Pre-ribosomal RNA processing is initiated by a cleavage in the 3′-ETS by the endoribonuclease Rnt1p. Following 3′-ETS processing, the 35S precursor is cleaved at A_0 and A_1, forming the 32S precursor. Cleavage at A_2 then splits the 32S precursor into the 20S and $27SA_2$ precursors. The 20S precursor is exported to the cytoplasm and cleaved at site D, leading to the mature 18S rRNA. The $27SA_2$ precursor is processed into 27SB precursor. The 27SB precursor is then cleaved at sites C_1 and C_2 to generate the mature 25S rRNA and the 7S precursor, which is then processed by the exosome into mature 5.8S rRNA. This figure is adapted from Kressler *et al*. (1) and Venema and Tollervey (2).

biogenesis, we have named the gene *ESF1* (Eighteen S rRNA Factor 1).

MATERIALS AND METHODS

Yeast strains, media and plasmids

The *S.cerevisiae* strains used for phenotypic analysis in this study are R1158 (wild type) (14) and TH_2070, the *tetO7-ESF1* mutant (10). The vector pBS1479 (15) was used to C-terminally tag Esf1p in strain YCK245 (a derivative of W303-1A) (16), creating strain YNJK654. pRS411 (a plasmid containing *MET15*) was a gift from Jef Boeke. All yeast cultures were at 30°C. Yeast transformation was carried out according to a protocol described previously (17). Rich medium YPD (1% yeast extract, 2% peptone, 2% dextrose) (18) was used to culture yeast for protein purification. Synthetic medium SD (18) was used to culture yeast for RNA isolation and pulse–chase labeling.

RNA extraction and northern blotting

For depletion of Esf1p protein, *tetO7-ESF1* (and the isogenic wild-type control strain) was exposed to 10 μg/ml doxycycline (Sigma) for a total of 24 h before harvesting for RNA extraction. RNA extraction and northern blotting were performed as described previously (10). Oligonucleotides specific for 35S pre-rRNA are: 18S, 5′-CAGAAGGAAAGGCCCCGTTGGAAATCCAGTACACGAAA AAATCGGACCGG-3′; 25S, 5′-TTCCCAAACAACTCGACTCTTCGAAGGCACTTTACAAAGAACCGCACTCC-3′; DA2, 5′-GAAAGAAACTTACAAGCCTAGCAAGACCGCGCACTTAAGCGCAGGCCCGG-3′; A2A3, 5′-TACCTCTGGGCCCCGATTGCTCGAATGCCCAAAGAAAAAGTTGCAAAGAT-3′; EC2, 5′-TCCAATGAAAAGGCCAGCAATTTCAAGTTAACTCCAAAGAGTATCACTCAC-3′; 5′-ETS-A0, 5′-GGAAATGCTCTCTGTTCAAAAAGCTTTTACACTCTTGACCAGCGCACTCC-3′. Oligonucleotides for snoRNAs are: U3, 5′-CCCTATCCCTTCAAAAAAGAAGTACATAGGATGGGTCAAGATCATCGCGC-3′; U14, 5′-GCGGTCACCGAGAGTACTAACGA-3′; snR10, 5′-CACATTCTTCATGGGTCAAGAACGCCCCGG-3′; snR30, 5′-TCCATATATATCATGGCAACAGCCCCCGAA-3′.

Pulse–chase labeling

R1158 (wild type) and *tetO7-ESF1* mutant were transformed with pRS411 and grown to 0.8×10^7 cells/ml over a period of

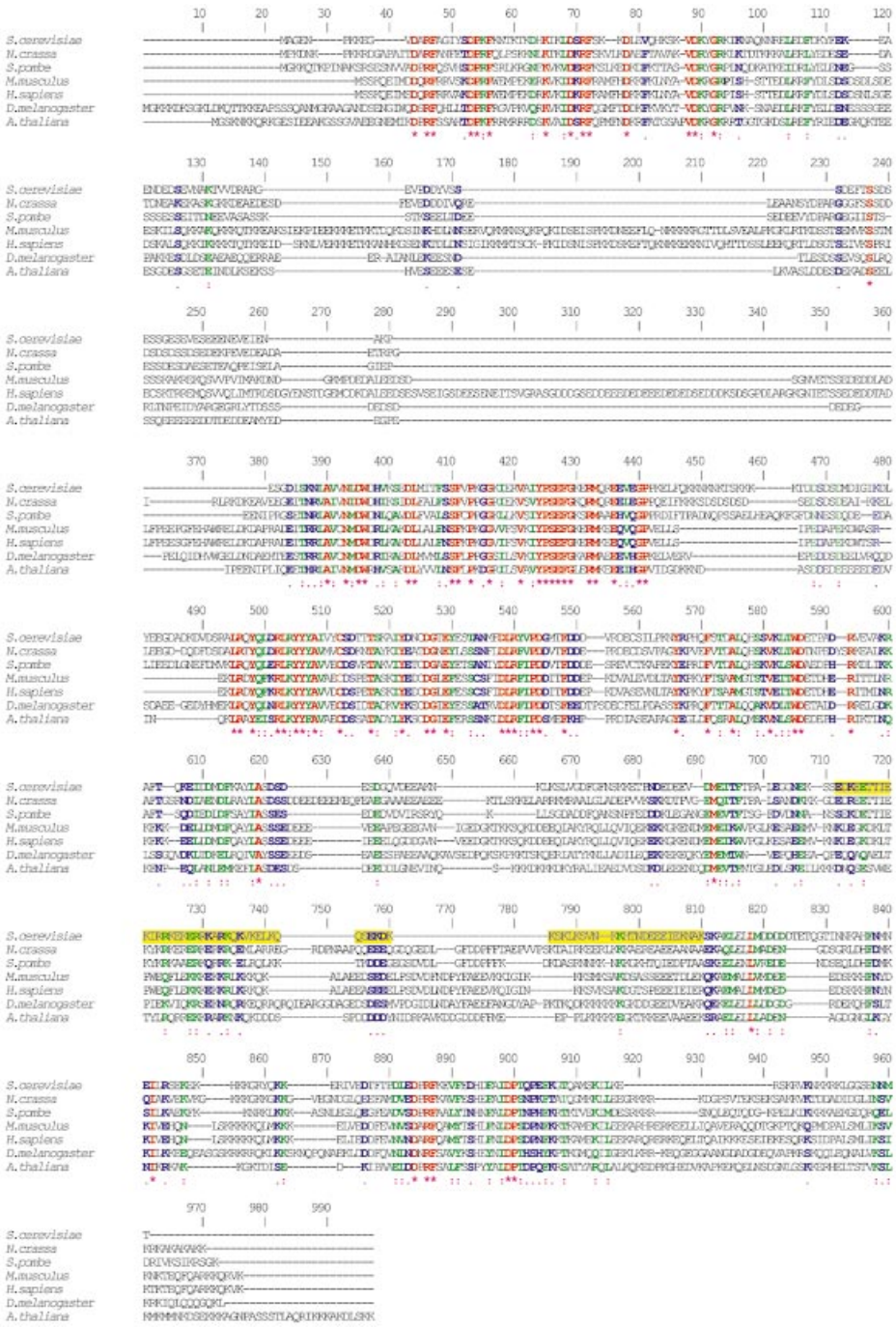

Figure 2. Amino acid alignment of Esf1p with homologs from other organisms. Sequence alignments were carried out using the CLUSTAL W program. Convention was taken as follows: red (asterisk), all identical residues; green (colon), almost identical residues; and blue (period), similar residues. Accession numbers for the sequences are as follows: *S.cerevisiae*, NP_010653; *N.crassa*, EAA27818.1; *S.pombe*, NP_595418; *M.musculus*, XP_130548; *H.sapiens*, NP_057733.2; *D.melanogaster*, AAM52674.1; *A.thaliana*, AAF26158. The yellow shaded region is the coiled-coil domain of Esf1.

24 h in SD-Met medium containing 10 μg/ml doxycycline. Pulse–chase labeling was performed as described by Kressler *et al*. (19) with minor modifications. Aliquots comprising of 20 000 c.p.m. were loaded on each lane of a 1% agarose/glyoxal gel and RNA was transferred to a Hybond-N$^+$ nylon membrane (Amersham Pharmacia) by overnight downward capillary transfer. Membranes were sprayed with EN^{3}HANCE (PerkinElmer Life Sciences), dried, and exposed to X-ray films at –80°C with an intensifying screen and visualized by autoradiography.

Affinity purification and mass spectrometry analysis

Four liters of YNJK654 cells were grown in YPD to 1.5×10^7 cells/ml, then harvested. The cell pellets were washed twice with cold water and frozen with liquid nitrogen. Frozen cell pellets were broken with dry ice in a small coffee grinder

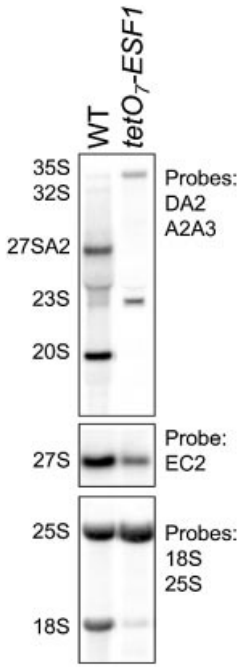

Figure 3. Northern blot analysis shows that *ESF1* is required for pre-rRNA processing at sites A_0, A_1 and A_2. Equal amounts (5 µg) of total RNA from each strain were resolved on a 1% agarose/glyoxal gel, blotted to a nylon membrane, and probed sequentially with oligonucleotides as indicated.

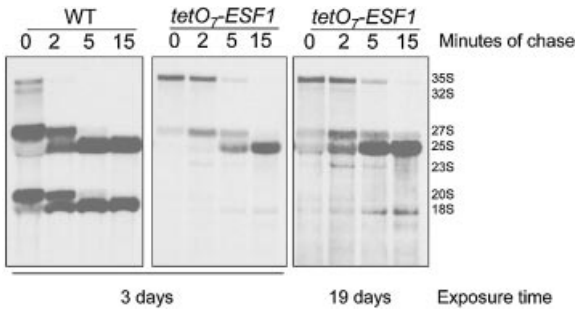

Figure 4. Pulse–chase analysis shows that depletion of Esf1 affects the biogenesis of 18S rRNA. Cells were pulsed with [methyl-^{3}H]methionine and chased for up to 15 min with an excess of unlabeled methionine. Total RNA was extracted and aliquots comprising of 20 000 c.p.m. were loaded on each lane of a 1% agarose/glyoxal gel. The positions of the pre-rRNAs and mature RNAs are indicated.

(Krups), and 10 ml IPP150 buffer (10 mM Tris pH 8.0, 150 mM NaCl and 0.1% Triton X-100) plus 1 mM DTT and protease inhibitors were added to the lysed cells. The broken cells were subjected to centrifugation at 13 000 r.p.m. in an SS-34 rotor (20 200 *g*) for 1 h at 4°C. The lysates were mixed with 200 µl IgG agarose beads for 2 h at 4°C. After five washes with 0.5 ml IPP buffer, the IgG beads were collected and the RNA associated with the beads extracted with hot phenol and RNase-free DNase I was used to remove DNA in the samples as described (10). For the purification of protein complexes, the eluates from IgG columns were further purified with calmodulin as previously described (16). The purified proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on gels containing 10% polyacrylamide, visualized by silver staining and identified by MALDI-TOF mass spectrometry.

RESULTS

Esf1p is an essential, evolutionarily conserved, nucleolar protein

ESF1 encodes a protein of 628 amino acids with a calculated molecular weight of 72.4 kDa and pI of 4.99. The encoded Esf1 polypeptide has a coiled-coil region located between residue 426 and 492, and a bipartite nuclear localization signal near its N-terminus (from residue 447 to 464). We identified clear sequence counterparts in other organisms, including *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, *Drosophila melanogaster*, *Neurospora crassa*, *Drosophila melanogaster*, *Mus musculus* and *Homo sapiens*. The multiple sequence alignment reveals what appear to be several discrete conserved regions (Fig. 2). In a systematic deletion analysis, Giaever and colleagues (20) reported that *ESF1* is essential for viability. The protein has been localized to the nucleus, possibly enriched in the nucleolus, by three independent studies (21–23).

ESF1 is required for 18S rRNA synthesis and for cleavage of the pre-rRNA at sites A_0, A_1 and A_2

Our previous microarray study indicated that the *tetO$_7$-ESF1* mutant had a defect in 18S rRNA biogenesis (10). To investigate this further, we analyzed the steady-state levels of mature and precursor rRNA molecules by northern blotting. Probes specific for mature 18S and 25S rRNAs confirmed that the level of 18S rRNA was dramatically reduced upon depletion of Esf1p, relative to 25S rRNA (Fig. 3). Hybridizing the membrane with oligonucleotides complementary to D-A_2 and A_2-A_3 regions (probes DA2 and A2A3) revealed that 20S pre-rRNA (the direct precursor to 18S rRNA) and the 27SA_2 pre-rRNA (normally a precursor to the 25S rRNA), were not detected in the *tetO$_7$-ESF1* mutant. We also observed accumulation of 35S pre-rRNA and appearance of aberrant 23S pre-rRNA in the mutant. The 23S pre-rRNA is generated by cleavage at A_3 in the 35S precursor without the early cleavages at sites A_0, A_1 and A_2 (2). These data indicate that *ESF1* is required for pre-rRNA processing at sites A_0, A_1 and A_2 that lead to production of the mature 18S rRNA.

To further confirm that depletion of Esf1p affects pre-rRNA processing, we carried out pulse–chase labeling of the pre-rRNA with [methyl-^{3}H]methionine. In wild-type cells, the 35S precursor was processed into 25S and 18S rRNAs in ~5 min (Fig. 4). In contrast, *tetO$_7$-ESF1* mutant contained very little 20S pre-rRNA and 18S rRNA at any time point examined (up to 15 min). The formation of 25S rRNA was delayed, but it was produced much more rapidly than 18S rRNA. Processing of the 35S precursor was also delayed in the Esf1p-depleted strain, perhaps because cleavage at A_3 in the absence of cleavage at A_2 is not the primary pathway. This would also explain the delay in 25S formation (Fig. 4), and the slight reduction in steady-state 27S levels (Fig. 3).

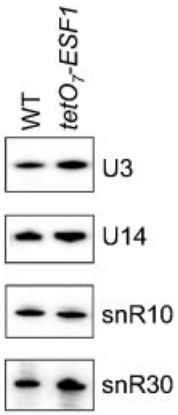

Figure 5. Northern blot analysis shows that *ESF1* does not affect levels of Box C/D snoRNAs U3 and U14 or Box H/ACA snoRNAs snR10 and snR30. Equal amounts (5 μg) of total RNA from each strain were separated on an 8% polyacrylamide/TBE/urea gel, blotted to a nylon membrane and hybridized with probes as indicated.

Depletion of Esf1 does not affect the steady-state levels of box C/D snoRNAs U3, U14 and H/ACA snoRNAs snR10, snR30

To assess whether Esf1 is involved in the biogenesis or maintenance of the snoRNAs U3, U14, snR10 and snR30 [required for cleavage at A_0, A_1 or A_2 (3,7–9)], the steady-state levels of each RNA were monitored in the *tetO7-ESF1* mutant strain. As seen in Figure 5, the *in vivo* levels of these RNAs in the *ESF1* mutant were similar to wild type. This suggests that ESF1 is not involved in biogenesis or stability of these snoRNAs.

Esf1p associates with ribosomal proteins, proteins required for small-subunit biogenesis and U3 and U14 snoRNAs

Using the TAP tag (15), we affinity-purified Esf1p to ascertain which proteins and RNAs were physically associated. Esf1p–TAP co-purified with a number of proteins involved in ribosome biogenesis (Fig. 6), including Nsr1p, Krr1p, Nop1p, Utp22p and Puf6p, all of which are involved in 18S rRNA synthesis (2,5,24–26). In addition, Esf1p–TAP purified with a number of proteins that are components of the ribosome (Rps1p, Rps3p, Rps4p, Rps6p, Rps11p, Rpl2p, Rpl3p, Rpl4p, Rpl7p, Rpl10p, Rpl20p and Rpp0p).

To assay RNAs associated with Esf1p–TAP, RNA was phenol-extracted directly from washed IgG beads. As positive controls, we also affinity-purified Utp18p–TAP, Utp22p–TAP and Nop58p–TAP, which are known to be associated with the U3 snoRNA (2,4,5). Northern blotting revealed that Esf1p–TAP, as well as the three positive controls, co-purified with U3 and U14 snoRNAs (Fig. 7). The protein–RNA associations were stable after washes in 400 mM NaCl. In contrast, TAP-purifications of two other proteins (Krr1p and Nop14p), which are also involved in 18S rRNA biogenesis, did not contain U3 and U14 snoRNAs in the precipitates, in agreement with previous observations (5). Other negative controls (a wild-type strain with no tag and other unrelated proteins Ths1p–TAP, Pus1p–TAP and Gln1p–TAP) also failed to precipitate appreciable amounts of these RNAs. We also detected U14 snoRNA in the Utp18p–TAP and Utp22p–TAP pulldowns, even under very high stringent tandem affinity purifications in which a high-speed centrifugation step was added to the TAP procedure (5), which we note conflicts with previously published observations (4).

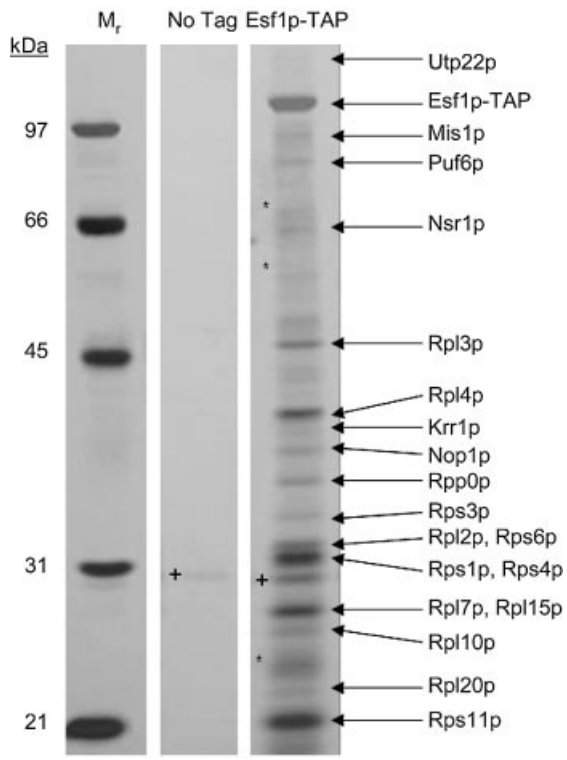

Figure 6. TAP purification of Esf1p. Purification of Esf1p was carried out with strains containing either no tagged protein or a TAP-tagged version of Esf1p. After the protein complex was purified, it was analyzed by SDS–PAGE and silver staining. Esf1p and other proteins were identified by trypsin digestion and MALDI-TOF mass spectrometry. The asterisks represent degradation products of Esf1p while the cross corresponds to TEV protease, used in the purification procedure (15).

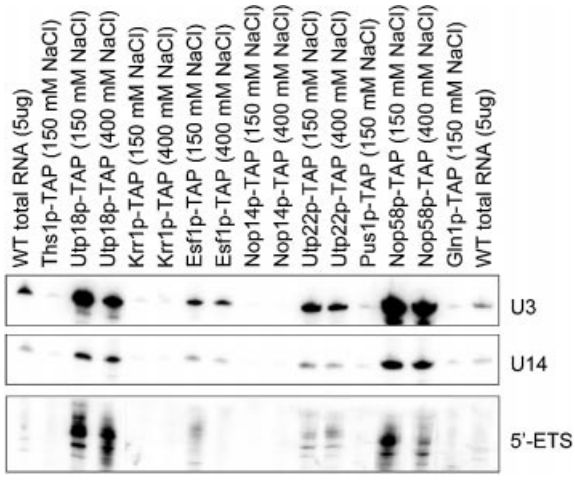

Figure 7. Northern blotting of RNA species co-purified with Esf1p–TAP. Total RNAs were extracted directly from washed IgG beads, separated on a 6% polyacrylamide/TBE/urea gel, transferred to a nylon membrane and hybridized with oligonucleotide probes as indicated. The same starting culture volume (4 l) was used for all lanes. We verified, for each purification, that the tagged protein was recovered (data not shown).

We also detected trace amount of 5′-ETS-A_0 and 5′-ETS-A_1 in the Esf1p–TAP precipitate (Fig. 7), which we have previously shown to associate with components of the SSU processome in the TAP purifications (5). However, we did not detect 20S precursor in the Esf1p immunoprecipitates (data not shown).

DISCUSSION

We present several lines of evidence that Esf1p is involved directly in 18S rRNA biogenesis. First, depletion of Esf1p led to a virtually complete loss of 20S and $27SA_2$ precursors, suggesting that the cleavage at site A_2 was blocked in the mutant (Fig. 3). In addition, the aberrant 23S RNA species and 35S precursor accumulated in the mutant, indicating inhibition of cleavage events at A_0, A_1 and A_2 (2). Although 18S rRNA biogenesis was affected in the mutant, 25S rRNA levels remained largely normal, suggesting that the 5.8S and 25S rRNAs are formed normally following cleavage at A_3 (Fig. 3). Studies of kinetics of rRNA formation in the mutant also suggested that Esf1p mainly affected the biogenesis of 18S rRNA (Fig. 4).

Among the proteins that co-purified with Esf1p, several are involved in 18S rRNA biogenesis. The nucleolar protein Nop1p is a component of the SSU processome complex (4). Nop1p is also a core component of all Box C/D snoRNPs (2), which include both U3 and U14 snoRNPs. Krr1p is required for 40S ribosome biogenesis (24) and subsequent export to the cytoplasm (5) and was found in the 90S pre-ribosome (5), although in our hands it does not co-purify with U3 or U14 RNAs, consistent with previous results (6) (Fig. 7). Nsr1p, a homolog of mammalian nucleolin (25,26), contains two RRMs (RNA recognition motifs) and a glycine/arginine-rich (GAR) domain. It is required for 18S rRNA synthesis (25,26). Utp22p is also involved in maturation of pre-18S rRNA (5,10) and has been described as a component of the SSU processome (27), although we have previously identified Utp22p in a distinct processome 'sub-complex' containing Rrp7p and yeast casein kinase (5). Aside from Utp22p and Nop1p, we did not detect association of Esf1p with any of the components of the SSU processome, indicating that it is not a core component of this large RNP.

Hazbun and colleagues previously identified an Esf1p-associated complex which differs dramatically from ours, containing Bfr2p, Enp2p, Hca4p, Lcp5p, Nop58p and Utp9p (21). Among these proteins, Hca4p, Lcp5p, Nop58p and Utp9p are involved in 18S rRNA synthesis (2,4,28,29). Hca4p is a putative DEAD box RNA helicase and over-expression of *HCA4* can suppress a U14 mutant (28). Lcp5p was found to be associated with U3 snoRNA (29) and Nop58p and Utp9p were found in both the SSU processome and 90S pre-ribosome (4,6), although Lcp5p was not found in the SSU processome (4). Bfr2p was also found in the 90S pre-ribosome (6). Hence, Hazbun and colleagues' data also support the general conclusion that Esf1p is involved in small-subunit biogenesis, although there is a complete lack of overlap with the specific associations we observed.

We propose that Esf1p-containing complexes may be dynamic. Although Esf1p co-purifies with two individual components of the SSU processome (Utp22p and Nop1p) (4,27) (Fig. 6), as well as the U3 and U14 snoRNAs and the 5′-ETS of the rRNA (Fig. 7), all of the associations appear to be sub-stoichiometric, with the possible exception of the ribosomal proteins themselves. This is also clearly the case for the complex described in Hazbun *et al*. (21). Dynamic complexes might explain why different laboratories obtain quite different results from affinity purifications, since the associated proteins might depend upon the growth phase at harvest, the extraction procedure and the purification protocol used. The presence of Esf1p in multiple pre-ribosomal processing intermediates might also underlie its association with protein components of both the large and small ribosomal subunits. Analysis of mutants in the conserved features of Esf1p (Fig. 2) might help resolve the biochemical role of Esf1p in 18S biogenesis: presumably, they represent discrete domains that mediate physical interactions and/or catalytic functions.

ACKNOWLEDGEMENTS

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