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Saccharomyces cerevisiae Sof1p Associates with 35S Pre-rRNA Independent from U3 snoRNA and Rrp5p

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Sof1p is a *trans*-acting protein that is essential for biogenesis of the 40S ribosomal subunits in the yeast *Saccharomyces cerevisiae*. Because of its involvement in the early cleavage steps of precursor rRNA, its interaction with Nop1p and its ability to coprecipitate U3 snoRNA, Sof1p has has so far been regarded as a protein that is specific to the U3 snoRNP. To determine whether a site exists within U3 snoRNA with which Sof1p directly or indirectly associates, we studied the ability of ProtA-tagged Sof1p to coimmunoprecipitate mutant versions of U3 snoRNA. None of the tested mutations had a significant effect on the recovery of mutant U3 from cell extracts. Further coimmunoprecipitation experiments, using cells that could be genetically depleted for either Sof1p or U3 snoRNA demonstrated that the two factors associate independently of each other with the 35S precursor RNA. Indeed, association between Sof1p and U3 snoRNA was abolished in cells in which 35S pre-rRNA transcription was blocked. Finally, we found that an overall reduction in the levels of box C/D snoRNPs by genetic depletion of the common Nop58p protein did not affect coprecipitation of 35S pre-rRNA by Sof1p. From these data, we conclude that Sof1p does not assemble into the 90S preribosome as part of the U3, or any other box C/D, snoRNP. The early and independently assembling *trans*-acting factor Rrp5p also proved to be dispensable for assembly of Sof1p.

The production of eukaryotic ribosomes is a highly dynamic process that starts in the nucleolus, a subregion of the nucleus. Here, the 18S, 5.8S, and 25-28S rRNA genes are transcribed into a single large precursor rRNA by RNA polymerase I, while transcription of the 5S rRNA genes by RNA polymerase III gives rise to a separate pre-5S rRNA transcript. Most of the processing of these precursors into the mature rRNA species and their assembly with ribosomal proteins also occurs in the nucleolus.

The RNA polymerase I transcript (35S pre-rRNA in yeast) contains, in addition to the three mature rRNAs, two external (5′ and 3′ external transcribed spacer [ETS]) and two internal (internal transcribed spacer 1 [ITS1] and ITS2) transcribed spacers (Fig. 1). Removal of the spacer elements involves an ordered series of endo- and exonucleolytic cleavages (for reviews see references 19, 24, and 31). Large complexes containing different sets of *trans*-acting proteins, ribosomal proteins, and small nucleolar RNAs (snoRNAs) are formed on the various precursor rRNA species to guide this maturation, which has been studied in most detail in the yeast *Saccharomyces cerevisiae*. The earliest complex, formed on the yeast 35S precursor, is the 80S/90S preribosome. Within this particle, first the 5′ ETS is removed by cleavages at sites A0 and A1. Cleavage at site A2, or alternatively, at site A3, within ITS1 then separates the 90S preribosome into the 43S and 66S preribosomal subunits that contain the 20S and the 27SA2, or 27SA3, precursor rRNAs, respectively. The former particle is exported to the cytoplasm where the 20S pre-rRNA is matured into 18S rRNA in a single step. Further maturation of the 27SA2 pre-rRNA into 5.8S and 25S rRNA occurs in the nucleoplasm via two distinct pathways that give rise to 5.8S rRNA species that differ slightly in length at their 5′ ends (Fig. 1).

A large portion of the *trans*-acting factors involved in ribosome biogenesis consists of snoRNPs that can be divided into two functionally overlapping classes. Most snoRNPs act as guides in the nonessential, site-specific modification of the mature sequences within the 35S pre-rRNA, either by 2′-*O*-methylation or pseudouridylation (1, 11, 17, 18). A small set, however, which in yeast includes the U3, U14, snR10, and snR30 species as well as RNase MRP (mitochondrial RNA processing), is required for specific cleavage events. U14 and snR10 also act as modification guides, whereas U3 and snR30 do not have such a dual role.

U3 is the most abundant and best-studied snoRNP. It is absolutely required for the early cleavages at sites A0, A1, and A2, the first two of which are essential for formation of the 18S rRNA of the small subunit (15, 29). Using density gradient analysis, the U3 snoRNA can be detected in two complexes, the 90S preribosome and a much smaller complex sedimenting at 12S (8). The latter, often referred to as the U3 monoparticle, contains a set of proteins that are common to all box C/D snoRNPs, including Nop1p, Nop56p, Nop58p, and Snu13p, as well as the U3-specific protein Rrp9p (8, 30, 33, 35). The U3 monoparticle is recruited to the 35S pre-rRNA, where it assembles with some 30 additional proteins into the 80S/90S preribosome or small subunit (SSU) processome (6).

Genetic replacement of the yeast box C/D snoRNP-specific protein Nop1p by its human counterpart fibrillarin results in temperature-sensitive growth. In a screen to identify new *trans*-acting factors, a mutation within the gene *SOF1* (*suppressor of fibrillarin*) was identified that suppressed this phenotype (16).

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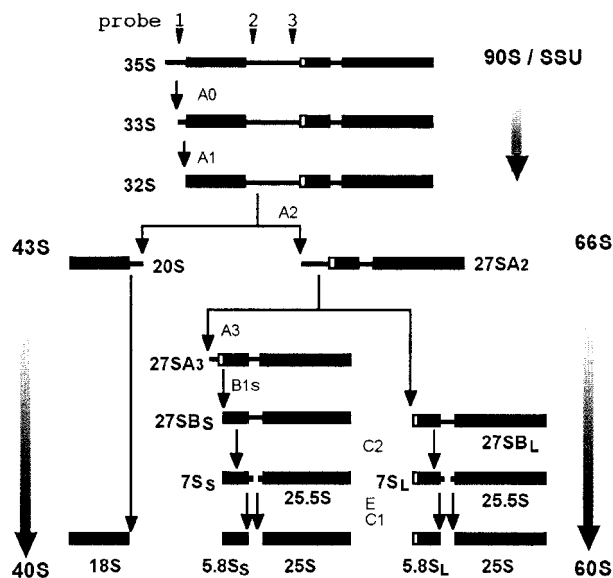
FIG. 1. Pre-rRNA processing in *Saccharomyces cerevisiae*. The mature 18S, 5.8S, and 25S rRNAs are shown as thick bars; transcribed spacer sequences are shown as thin bars. The processing sites and the locations of oligonucleotide probes 1, 2, and 3 used in primer extension and Northern analyses are depicted.

Further analysis showed that genetic depletion of Sof1p results in defective processing at sites A0 to A2 and that epitope-tagged Sof1p coprecipitated U3 but not U14 snoRNA, another member of the box C/D class. Consequently, Sof1p was considered to be a U3-specific protein.

In vivo coimmunoprecipitation studies reported in this paper show, however, that none of the U3 snoRNA-specific structural features is essential for its association with Sof1p, raising the question whether Sof1p is indeed a U3-specific protein. Therefore, we investigated the mutual dependence of U3 and Sof1p in their assembly with the early preribosome. Coimmunoprecipitation experiments in strains that could be genetically depleted for one or the other factor showed that Sof1p is still incorporated into the preribosome in the absence of U3 snoRNA and vice versa. On the other hand, inhibition of RNA polymerase I transcription abolished the association between Sof1p and U3, which, therefore, depends upon the presence of 35S pre-rRNA. These data demonstrate that Sof1p is not part of the U3 monoparticle and that U3 and Sof1p are assembled independently onto the 35S pre-rRNA. We also demonstrate that overall depletion of box C/D snoRNPs does not diminish the association of Sof1p with 35S pre-rRNA and that Sof1p does not require Rrp5p, another early assembling component of the early 90S preribosome, for this association.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* strain MH1 was used for cloning and propagation of plasmids. Yeast transformation was carried out according to the method of Gietz et al. (12). Yeast strains used in this work are listed in Table 1. The pRS series of plasmids is described by Sikorski and Hieter (27). A plasmid-encoded ProtA-tagged *SOF1* was constructed by cloning the 1.7-kb BamHI-Asp718 fragment (which includes the native *SOF1* promoter) and the 0.5-kb Asp718-HindIII fragment from pUN100-Sof1::ProtA (16) into pBS(KS+) cleaved with BamHI and HindIII. Next, the 2.3-kb Sof1::ProtA XbaI-XhoI fragment was ligated blunt ended into the BamHI site of pASZ11 (28). For co-immunoprecipitation experiments, the resulting pASZ11-Sof1::ProtA was transformed into JH84 (25) as well as NOY504 (23). We will refer to these strains as JH84 + pSof1::ProtA and NOY504 + pSof1::ProtA. pASZ11-ProtA::Rrp9 was constructed by cloning the 3.0-kb SacI-Asp718 ProtA::rrp9 fragment from pHIS3-ProtA::Rrp9 (33) into the corresponding sites of pASZ11. The resulting plasmid pASZ11-ProtA::Rrp9 was then transformed to JH84 and NOY504. YHV159 was constructed as described previously (34). pUN100-Sof1::ProtA (16) was introduced to YJV154 (32) and D499 (kind gift of David Tollervey) to create strains YJV154 + pSof1::ProtA and D499 + pSof1::ProtA, respectively.

Construction of the *GAL10::sof1* fusion gene. To construct the *GAL10::sof1* fusion gene, the *SOF1* gene was amplified from total yeast DNA by PCR, using a primer that introduces a BamHI site at the start codon and a second primer flanking the internal PstI site of *SOF1*. This BamHI-PstI subfragment was joined to the PstI-PstI *SOF1* subfragment from pUN100-*SOF1* (16) and cloned into the corresponding sites of pBluescript II KS+ (Stratagene). A 2.2-kb BamHI-BamHI fragment from this plasmid containing the complete *SOF1* gene was cloned blunt into the EcoRV site of pTL26 containing the *GAL1-10* promoter (20). A 3.0-kb XhoI-XbaI fragment encompassing the *GAL10::sof1* fusion gene was cloned in the corresponding sites of pRS315(LEU2). This construct was used for plasmid shuffling (see below).

Strain D344 (*sof1::HIS3*) containing a temperature-sensitive *SOF1* gene encoded on plasmid pUN100-LEU2 was transformed with pRS316(URA3)-Sof1::ProtA and incubated at 30°C on selective plates. Next, transformants were incubated at 37°C on nonselective yeast-peptone-dextrose plates to allow loss of pUN100(LEU2)-Sof1ts. Leu⁻ cells were transformed with pRS315(LEU2)-GAL10::sof1, grown on selective medium and streaked out on 5-fluoroorotic acid plates, to create strain YRB344.

The 3.0-kb SacI-Asp718 ProtA::Rrp9 fragment from pHIS3-ProtA::Rrp9 (33) was cloned into the corresponding sites of pRS314(TRP1) to create pRS314(TRP1)-ProtA::rrp9. YRB344 was transformed with pTRP1-ProtA::Rrp5 (7) to create YRB344 + pProtA::rrp5.

Immunoprecipitation, isolation, and analysis of RNA and proteins. Immuno-precipitations and Northern and Western analysis were carried out essentially as described previously (34).

RESULTS

U3 snoRNA-specific structural features are not essential for association with Sof1p in vivo. Since Sof1p was found to coprecipitate U3, but not U14, snoRNA and repressing Sof1p expression inhibited processing of pre-rRNA at sites A0 to A2, Sof1p was considered a specific protein component of the U3 snoRNP (16). To try to identify the structural element(s) within the yeast U3 snoRNA involved in its association with Sof1p, we introduced a plasmid-encoded Sof1-ProtA construct into yeast strain JH84, which carries a single U3 snoRNA gene under control of the repressible *GAL1-10* promoter (25). This strain was then transformed with different members of a set of previously constructed mutant U3 snoRNA genes (25) that carry various deletion or substitution mutations, as depicted in Fig. 2A. The substitution mutants also contain a tag inserted in hairpin 4, which enables specific detection of the mutant transcript (Fig. 2B, cf. lane 2 and 3). Transcripts of the deletion mutants were visualized using a probe that hybridizes to the

TABLE 1. Yeast strains used in this study

| Strain	Genotype	Reference
JH84	*MATα ade2-1 his3Δ leu2-3 ura3-52 can1-100 URA3-GAL::U3A U3B::LEU2*	25
NOY504	*MATα rpa12::LEU2 leu2-3,12 ura3-1 trp-1 his3-11 can1-100*	23
YJV154	*MATa ade2 his3 leu2 trp1 ura3 URA3-Gal::rrp5*	34
D499	*MAT? his3 leu2 ura3 trp1 gal2 galΔ108 HIS3-GAL::nop58*	19
D344	*MAT? his3 leu2 ura3 trp1 sof1::HIS3 + pLEU2-sof1-56*	16
YRB344	*MAT? his3 leu2 ura3 trp1 sof1::HIS3 + pRS315-GAL10::sof1 (CEN-LEU2)*	This work
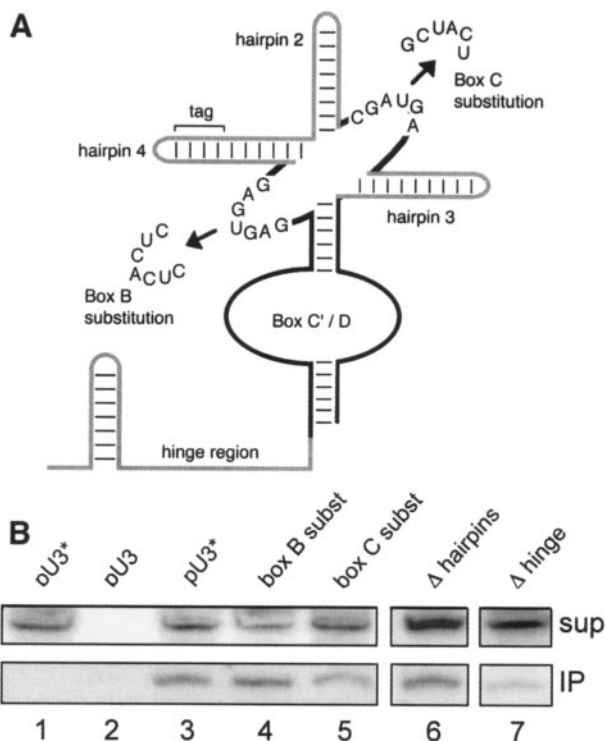
FIG. 2. Coimmunoprecipitation of U3 snoRNA carrying different mutations by ProtA-Sof1p. JH84 cells transformed with plasmids encoding the different mutant U3 species were grown on glucose-based medium for 24 h to deplete chromosomally encoded U3 snoRNA. Total cell extracts were prepared and treated with IgG beads. (A) Schematic representation of the yeast U3 snoRNA. A unique hybridization tag in hairpin 4 was used to distinguish between chromosomal and plasmid-borne (∗) U3 snoRNA (25). The base substitutions (subst) in boxes B and C are depicted, and the deletions of the hinge region and the hairpins 2, 3, and 4 are indicated by the gray lines. (B) Northern analysis of U3 snoRNA present in the supernatants (sup; upper panel) or precipitated material (IP; lower panel) using a probe complementary to the tag in hairpin 4 (lanes 1 to 5) or a probe the box C′ region (lanes 6 and 7).

box C′/D region and identified on the basis of altered electrophoretic mobility.

After depleting the JH84 cells for wild-type U3 by 24 h of growth on glucose-based minimal medium, total cell extract was mixed with immunoglobulin G (IgG)-agarose beads and the bound material was eluted with guanidine thiocyanate. Proteins and RNA were separated by phenol extraction and analyzed by Western and Northern hybridization, respectively. Similar amounts of tagged Sof1p were recovered from the extracts (not shown).

As shown in Fig. 2B (upper panel), all of the mutant U3 species are expressed at similar levels, confirming earlier results (25, 33). Northern analysis of the RNA retained by the beads (Fig. 2B, lower panel) shows that the substitution mutations in the boxes B and C of U3 snoRNA did not significantly affect the amount of these mutant snoRNAs coprecipitated by Sof1p-ProtA compared to the control experiment with wild-type (tagged) U3 (cf. lanes 4 and 5 to lane 3). The same holds true for the deletion encompassing hairpins 2 to 4 (lane 6). Mutant U3 snoRNA lacking the 5′-terminal region also re-

mains clearly detectable in the immunoprecipitate, though its level is diminished compared to the wild-type control (cf. lane 7 to lane 3).

Thus, none of the U3-specific structural features appear to be essential, either directly or indirectly, for association of Sof1p, leaving only the stem-loop-stem structure formed by the C′/D box and its flanking sequences as a possible region required for this association. Since the C′/D region is essential for stability of the U3 snoRNA (25), its importance for Sof1p association could not be tested by the in vivo approach used here. However, the fact that this structural feature is common to all box C/D snoRNPs, whereas Sof1p was found to coprecipitate only U3 snoRNA (16), makes it an unlikely candidate for direct recognition by the protein, although it remains formally possible that sequence differences between U3 and other snoRNAs in the flanking regions determine recognition of the former by Sof1p.

Association of U3 snoRNP and Sof1p with 35S pre-rRNA is mutually independent. Two possible scenarios for the assembly of Sof1p into the preribosome can be considered. In the first, Sof1p is incorporated independently of U3 snoRNA and coprecipitation of U3 snoRNA by Sof1p is the result of the presence of both factors in the 90S preribosome. Alternatively, Sof1p might be part of a complex whose formation depends on the C′/D box of U3 or another box C/D snoRNA. To try to distinguish between these possibilities, we first verified that Sof1p coprecipitates 35S pre-rRNA. Secondly, we tested whether Sof1p requires the U3 snoRNA for its stable association with 35S pre-rRNA. To this end, we expressed Sof1p-ProtA in strain JH84 (GAL::U3). Extracts were prepared from cells immediately before and 30 h after a shift from galactose- to glucose-based medium to deplete U3 snoRNP. Both extracts, as well as extracts from similarly treated, untransformed JH84 cells, were mixed with IgG-Sepharose beads, and the bound material was analyzed by Western and Northern hybridization. The presence of 35S pre-rRNA was determined by reverse transcription analysis, using a primer that hybridizes upstream from cleavage site A0 in the 5′ ETS (Fig. 1).

The data depicted in Fig. 3 show that the shift to glucose results in a severe reduction in U3 snoRNA (panel A, lanes 3 and 4). Depletion is not complete, however, because of the relatively high stability of this snoRNA (4). Treatment of extracts from galactose-grown cells with IgG beads confirms the association between Sof1p and U3 snoRNA (panel B, lanes 1 and 2) and also shows coprecipitation of significant amounts of 35S pre-rRNA (panel C). Depletion of U3 by the shift to glucose increases, rather than reduces, the amount of 35S precursor that is coprecipitated (panel C, lane 3) by the same amount of Sof1p, as determined by Western analysis (panel D, cf. lanes 1 and 3), despite the strong reduction in the level of coprecipitated U3 snoRNA (panel B, lane 3). From these data, we conclude that association of Sof1p with the 35S pre-rRNA does not depend upon the presence of U3, indicating that the protein is not incorporated into the early preribosome as a component of the U3 snoRNP. The increase in 35S pre-rRNA precipitated from extracts of U3-depleted cells can be explained by the accumulation of this precursor caused by the inhibition of processing at sites A0 to A2, which would lead to the recruitment of most or all of the cellular Sof1p into 90S preribosomes. The same phenomenon would also explain why we can still see co-
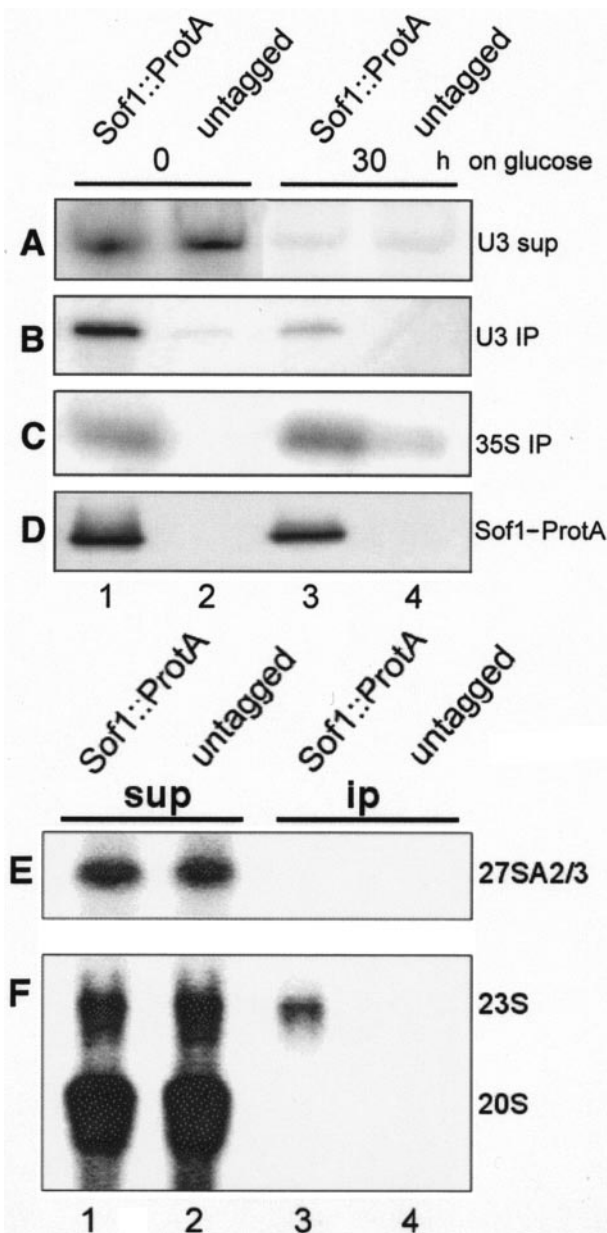
FIG. 3. Effect of depletion of U3 snoRNA on the association of 35S pre-rRNA with Sof1p. Total cell extracts were prepared from strain JH84 (*Gal*::*U3*) and JH84 carrying plasmid-borne Sof1::ProtA immediately before (lanes 1 and 2) and 30 h after (lanes 3 and 4) a shift from galactose- to glucose-based medium and treated with IgG beads. (A) Northern analysis of total RNA from the supernatants (sup) using the U3-specific probe that spans the box C′ region. (B) Northern analysis of total RNA from the coprecipitated (IP) material using the U3-specific probe. (C) Reverse transcription analysis of total RNA from the coprecipitated material using a primer complementary to the 5′ ETS upstream from cleavage site A0. (D) Western blot analysis of immunoprecipitated Sof1::ProtA. (E) Northern analysis of total and coprecipitated RNA using probe 3 to visualize 27SA2 and 27SA3 pre-rRNA. (F) Northern analysis of total and coprecipitated RNA using probe 2 to visualize 20S and 23S pre-rRNA.

precipitation of U3 snoRNA (panel C, lane 3), as the glucose-grown cells are expected to contain very little, if any, free U3 snoRNP.

To address whether Sof1p is associated with other pre-rRNAs, a Northern analysis was performed on total and immuno-
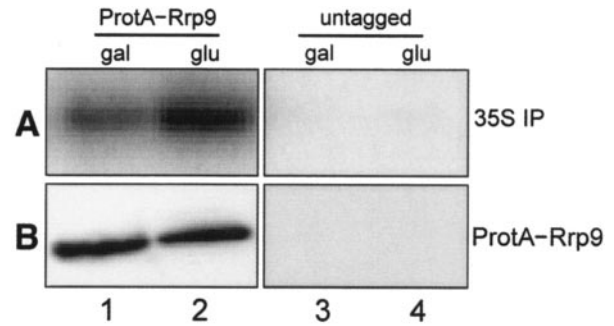

FIG. 4. Effect of Sof1p depletion on the association of U3 with 35S pre-rRNA. Total cell extracts were prepared from strain YRB344 (*Gal*::*sof1*) and YRB344 carrying plasmid-encoded ProtA-Rrp9p immediately before (lanes 1 and 3) and 24 h after (lanes 2 and 4) a shift from galactose (gal)- to glucose (glu)-based medium and treated with IgG beads. (A) Reverse transcription analysis of total RNA from the precipitated (IP) material using a primer complementary to the 5′ ETS upstream from site A0. (B) Western analysis of immunoprecipitated ProtA::Rrp9.

precipitated RNA obtained from JH84 cells grown on galactose that express Sof1p-ProtA (Fig. 3, panels E and F). Both the 20S and 27SA precursor species fail to be precipitated by Sof1p-ProtA, indicating that Sof1p leaves the 90S preribosome upon or immediately after its separation into 43S and 66S particles. On the other hand, we do see a clear association of Sof1p with 23S pre-rRNA, similar to what has been reported for other SSU processome components (3).

To determine whether Sof1p might be required for recruitment of the U3 snoRNP, a similar experiment was performed using strain YRB344, a derivative of D344 (16), in which the genomic *SOF1* gene has been disrupted and replaced by a plasmid-borne copy under control of the *GAL1-10* promoter. In addition, this strain carries a plasmid encoding a ProtA-tagged version of Rrp9p, a bona fide U3-specific protein (33), to allow immunoprecipitation of the U3 snoRNP.

YRB344 cells were shifted from galactose- to glucose-based medium to deplete them of Sof1p. That this treatment has the expected effect could be seen from the significant reduction in 18S rRNA levels in glucose-grown cells, while the level of 25S rRNA remained unaffected (data not shown) (cf. reference 16). Total cell extract was prepared from cells harvested immediately before and 24 h after the shift and subjected to immunoprecipitation as described above. Figure 4B shows that the level of tagged Rrp9p remains unaffected by the shift (cf. lanes 1 and 2). The results, depicted in Fig. 4A, show that the amount of 35S pre-rRNA that is specifically coprecipitated by tagged Rrp9p does not diminish upon depletion of Sof1p but, in fact, increases (cf. lanes 2 and 3), as in the case of U3 depletion described above. Because Rrp9p is an integral protein of the U3 snoRNP (33), we conclude that U3 becomes stably associated with the pre-rRNA, irrespective of the presence or absence of Sof1p.

In summary, the data presented in this section demonstrate that association of Sof1p and U3 snoRNP with 35S pre-rRNA, which indicates their incorporation into the 80S/90S preribosome, are independent events. Together with the results described in the previous section, we conclude that the classification of Sof1p as a U3-specific protein is incorrect.
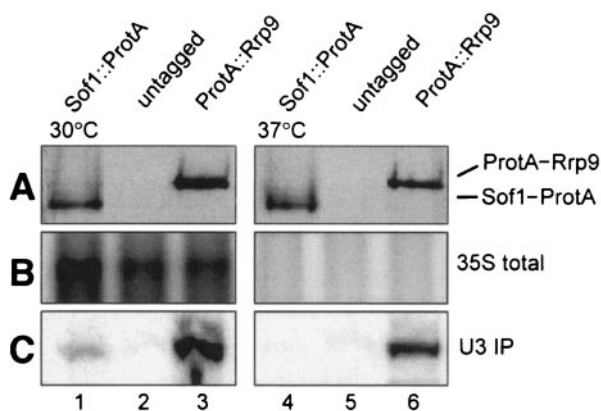
FIG. 5. Effect of blocking RNA polymerase I transcription on the association of U3 snoRNP and Sof1p. Total cell extracts were prepared from strains NOY504 (*rpa12::LEU2*), NOY504 carrying plasmid-encoded Sof1p-ProtA, and NOY504 carrying plasmid-encoded ProtA-Rrp9p immediately before (lanes 1 to 3) and 8 h after (lanes 4 to 6) a shift from 30°C to 37°C and treated with IgG beads. (A) Western blot analysis of the precipitated material. (B) Northern analysis of total RNA present in the original extracts using a probe complementary to the 5′ ETS upstream from site A0. (C) Northern analysis of total RNA from the precipitated (IP) material using a probe complementary to the C′ region of U3 snoRNA.
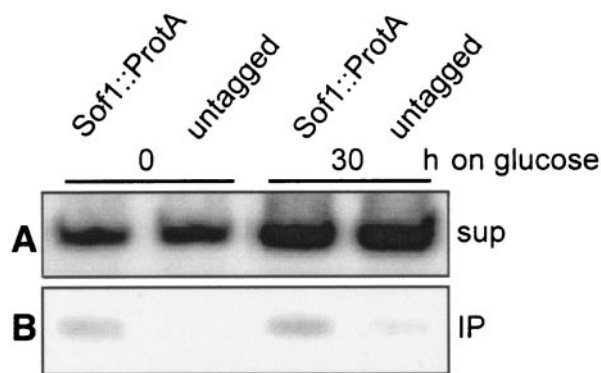

FIG. 6. Coimmunoprecipitation of U14 snoRNA by Sof1p-ProtA. Total cell extracts were prepared from strain JH84 (*Gal::U3*) and JH84 carrying plasmid-encoded Sof1::ProtA immediately before (lanes 1 and 2) and 30 h after (lanes 3 and 4) a shift from galactose- to glucose-based medium and treated with IgG beads. (A) Northern analysis of total RNA from the supernatant (sup) fractions using a probe complementary to the U14 snoRNA. (B) Northern analysis of the precipitated (IP) material using a probe complementary to the U14 snoRNA. The same extracts as analyzed for the results shown in Fig. 3 were used.

35S pre-rRNA is essential for association of U3 snoRNP and Sof1p. Since, Sof1p and U3 snoRNP associate with pre-rRNA as separate components, their coprecipitation should be dependent on the presence of the 35S precursor. Therefore, we examined coprecipitation of U3 snoRNA by tagged Sof1p in strain NOY504 that had been transformed with the plasmid encoding Sof1-ProtA. Strain NOY504 contains a deletion in the *RPA12* gene, which encodes a subunit of RNA polymerase I that is not essential at lower temperatures but is absolutely required for its activity at 37°C (23). As a control, we used the same parent strain transformed with a plasmid encoding ProtA-Rrp9p.

As shown in Fig. 5A, similar quantities of Sof1p-ProtA and ProtA-Rrp9p were recovered from the respective NOY504 transformants before and after the temperature shift (panel A). At 37°C, the production of 35S pre-rRNA is abolished (panel B, lanes 4 to 6). At the same time, coprecipitation of U3 snoRNA by tagged Sof1p is completely lost (panel C, lane 4), although the total amount of U3 snoRNA in the cells, as determined by precipitation with tagged Rrp9p, does not change (cf. lane 6 to lane 3). These results further support our conclusion that Sof1p, in contrast to Rrp9p, is not part of the U3 snoRNP monoparticle but that their association as detected by immunological assays, is solely the result of both components being part of the 90S preribosome. Of course, this does not exclude the possibility of a direct interaction of the two factors subsequent to assembly into the 90S particle.

Overall depletion of box C/D snoRNAs does not affect Sof1p-35S pre-rRNA association. Recently, it has been proposed that Sof1p and Nop1p together enter the nucleus through the nuclear pore by forming a trimer complex with Kap121p, a member of the karyopherin family (22). This physical interaction between Sof1p and Nop1p (a common component of box C/D snoRNPs), coupled with the fact that Sof1p genetically inter-

acts with Nop1p (16), suggest that, like Nop1p, Sof1p might interact with multiple box C/D snoRNPs. Therefore, we tested the Sof1-ProtA immunoprecipitate from strain JH84 analyzed as shown in Fig. 3 for the presence of U14 snoRNA. Cellular levels of U14 snoRNA were found to increase slightly upon depletion of U3 snoRNA (Fig. 6A, cf. lanes 3 and 4 to lanes 1 and 2). Surprisingly, in stark contrast to previous observations (16), we were able to detect significant amounts of U14 snoRNA in the Sof1p-precipitated material (cf. Fig. 6B, lane 1). The ability of Sof1p to coprecipitate U14 snoRNA is not affected by depletion of U3 snoRNA upon a shift to glucose (Fig. 6B, lane 3). This observation raises the possibility that Sof1p is assembled into the early preribosome as part of the U14 snoRNP and/or possibly another member of the box C/D family. We therefore introduced the *sof1::ProtA* gene into strain D499 carrying the conditionally expressible *GAL::nop58* gene. Nop58p is a core protein of box C/D snoRNPs that is essential for their stability (21). Depletion of Nop58p, therefore, causes a substantial overall reduction in the levels of box C/D snoRNAs. The parental strain D499 was used as a control. As shown in Fig. 7A, the level of Sof1p-ProtA is not affected by the depletion of Nop58p. Northern analysis reveals that, as previously observed (21), the cellular level of U14 snoRNA is significantly reduced by the depletion of Nop58p (Fig. 7B), as was the level of U3 snoRNA (data not shown). The amount of 35S pre-rRNA coprecipitated by Sof1-ProtA remains the same, however (Fig. 7C, cf. lane 3 to lane 1). This is clear evidence that stable association of Sof1p with the early preribosome is not compromised when the levels of all box C/D snoRNPs, including U3 and U14, are drastically reduced.

Assembly of Sof1p and Rrp5p into the preribosome is mutually independent. Rrp5p is a *trans*-acting factor that, like U3 snoRNA and Sof1p, is involved in processing at site A0 to A2, but it is also essential for cleavage at site A3 (32). We recently demonstrated that the putative RNA helicase Rok1p depends upon Rrp5p for its assembly into the preribosome (34). Since Rrp5p is among the earliest associating *trans*-acting factors (6),
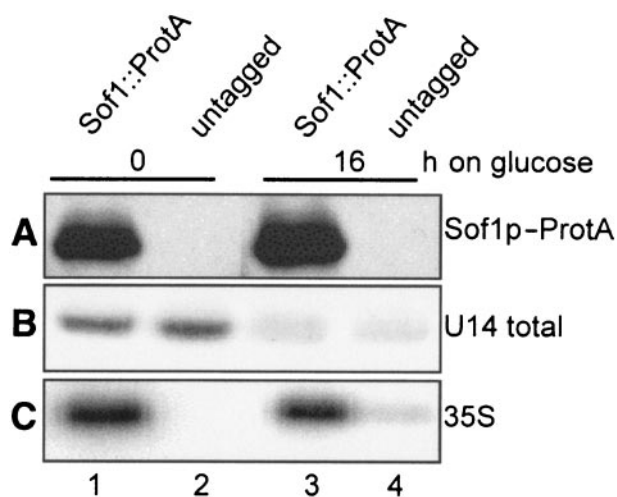
FIG. 7. Effect of Nop58p depletion on the association of Sof1p with 35S pre-rRNA. Total cell extracts were prepared from strain D499 (*Gal::nop58*) carrying plasmid-encoded Sof1::ProtA immediately before (lanes 1 and 2) and 16 h after (lanes 3 and 4) a shift from galactose- to glucose-based medium. (A) Western blot analysis of the precipitated material. (B) Northern analysis of total RNA from the original cell extracts using a probe specific for U14 snoRNA. (C) Reverse transcription analysis of total RNA from the precipitated material using a probe complementary to the 5′ ETS upstream from site A0.

we decided to analyze whether this protein is involved in incorporation of Sof1p into the 90S preribosome. To that end, we used strain YJV154 which contains the genomic *RRP5* gene under control of the *GAL1-10* promoter (32). This strain was transformed with a *sof1p::protA* fusion construct to assess whether Sof1p remains able to coimmunoprecipitate 35S pre-rRNA upon depletion of Rrp5p.

Extracts were prepared from cells grown on galactose-based medium and from an equal amount of cells cultured for 24 h after shifting them to glucose-based medium. After treatment with IgG-agarose beads, the precipitated material was analyzed by Northern analysis and reverse transcription.

Both in the presence and absence of Rrp5p, U3 snoRNA and 35S pre-rRNA are coprecipitated by tagged Sof1p (Fig. 8B and C, lanes 1 and 2). This confirms that the U3 snoRNP associates independently of Rrp5p (34) and shows that stable association of Sof1p with 35S pre-rRNA does not require Rrp5p. The slight increase in the amount of coprecipitated 35S pre-rRNA seen upon depletion of Rrp5p can again be explained by the inhibition of the early processing cleavages (see above).

Finally, we analyzed whether depletion of Sof1p has an effect on the assembly of Rrp5p into the preribosome using strain YRB344 (*GAL::sof1*) transformed with a plasmid that encodes the ProtA::Rrp5p fusion protein. Extracts from YRB344 cells harvested immediately before and 24 h after a shift from galactose- to glucose-based medium were subjected to immunoprecipitation as described above. As shown in Fig. 9B and C, ProtA::Rrp5p is able to coprecipitate U3 snoRNA and 35S pre-rRNA irrespective of the presence of Sof1p in the extract (cf. lanes 1 and 2). Thus, lack of Sof1p does not prevent association of Rrp5p with the 35S pre-rRNA.
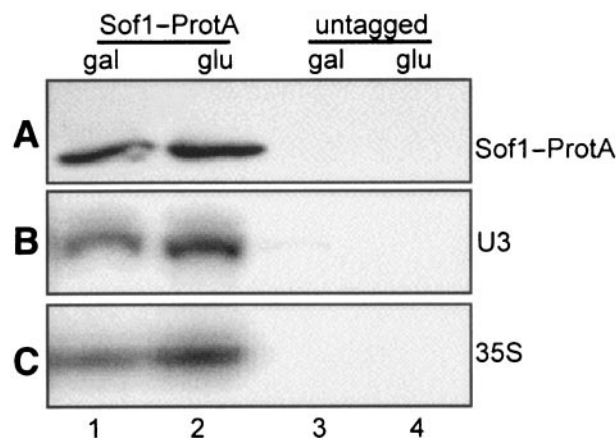

FIG. 8. Effect of Rrp5p depletion on the association of Sof1p with 35S pre-rRNA. Total cell extracts were prepared from strain YJV154 (*Gal::rrp5*) and strain YJV154 carrying plasmid-encoded Sof1::ProtA immediately before (lanes 1 and 3) and 24 h after (lanes 2 and 4) a shift from galactose- to glucose-based medium and treated with IgG beads. (A) Western blot analysis of the original extracts. (B) Northern analysis of total RNA from the precipitated material using a probe complementary to the C′ region of U3 snoRNA. (C) Reverse transcription analysis of total RNA from the precipitated material using a primer complementary to the 5′ ETS upstream from site A0.

DISCUSSION

Biogenesis of rRNA is a highly dynamic process that requires ordered assembly with and dissociation from the various pre-rRNA species of over 170 *trans*-acting factors and ribosomal proteins, giving rise to a series of preribosomal complexes. While over the past few years there has been considerable progress in establishing the order and composition of the different intermediate preribosomal particles (see refer-
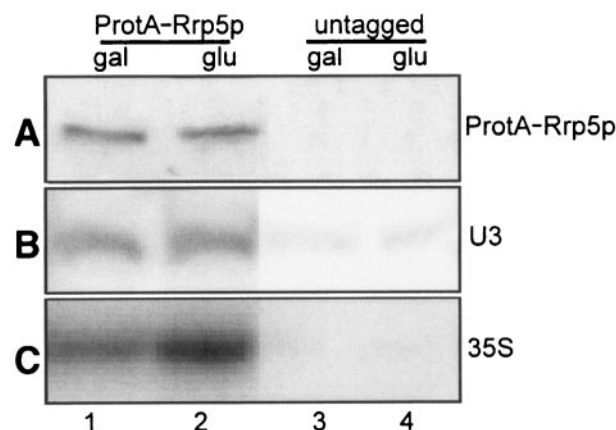

FIG. 9. Effect of Sof1p depletion on the association of Rrp5p with 35S pre-rRNA. Total cell extracts were prepared from strain YRB344 (*Gal::sof1*) and YRB344 carrying plasmid-encoded ProtA::Rrp5 immediately before (lanes 1 and 3) and 24 h after (lanes 2 and 4) a shift from galactose (gal)- to glucose (glu)-based medium and treated with IgG beads. (A) Western blot analysis of the original extracts. (B) Northern analysis of total RNA from the precipitated material using a probe complementary to the C′ region of U3 snoRNA. (C) Reverse transcription analysis of total RNA from the precipitated material using a primer complementary to the 5′ ETS upstream from site A0.

ence 24 for a recent review), relatively little is known as yet concerning the mechanisms and interactions than govern their formation. Coimmunoprecipitation experiments have proven to be a valuable tool in filling this gap in our knowledge (3, 5, 9, 10, 14, 26, 34). Here, we have used this approach to study Sof1p, a *trans*-acting factor that is required for maturation of 18S rRNA (16). In agreement with its involvement in the early processing cleavages at site A0 to A2, Sof1p is found in the 90S preribosome, also called the small subunit processome or SSU (6, 13). On the other hand, Sof1p has been reported to be associated specifically with U3 snoRNA (16). However, a search for sequences within U3 snoRNA that are essential for Sof1p association, by testing mutant U3 snoRNAs for their ability to be coimmunopurified with Sof1p showed that each of the U3-specific structural features of the snoRNA can be removed without abolishing its association with the protein (Fig. 2). The only mutant showing an effect at all, is the one carrying a deletion of the 5′-terminal region (Δhinge). Despite the fact that this mutant is expressed at about the same level as all the others (25) (Fig. 2B), the amount recovered from the co-precipitate was lower (Fig. 2C, lane 7). We ascribe this effect to a less stable interaction of U3 with the 35S pre-rRNA, in which base pairing of sequences in the hinge region with the 5′ ETS are known to play a crucial role (2, 4).

These results threw doubt upon the classification of Sof1p as a U3-specific protein, which were further strengthened by our observation that, in contrast to an earlier report (16), tagged Sof1p also coprecipitated the U14 snoRNA (Fig. 6B). Therefore, we decided to investigate the interdependence of these components during their incorporation into the early pre-ribosome. To that end, we first performed immunoprecipitation experiments using cells depleted for either U3 snoRNA (Fig. 3) or Sof1p (Fig. 4). In both cases, we found that the depletion of one factor slightly increased, rather than decreased, the amount of 35S pre-rRNA coprecipitated by the other factor. These findings constitute convincing evidence that Sof1p and U3 assemble as independent components into the 90S preribosome in which the 35S pre-rRNA acts as a platform. This conclusion is further supported by our observation that, in the absence of PolI transcription, the mutual coprecipitation of U3 snoRNA and Sof1p is abolished (Fig. 5). Thus, contrary to the earlier conclusion (16), Sof1p is not a stable component of the U3 snoRNP monoparticle. Our observation that Sof1p coprecipitates substantial amounts of the box C/D snoRNA U14 (Fig. 6B) together with the fact that Sof1p interacts genetically with the common box C/D protein Nop1p suggested a possible role for other box C/D snoRNAs in the association of Sof1p with the preribosome. An overall reduction in the levels of box C/D snoRNPs by genetic depletion of Nop58p, however, did not have a negative effect on coprecipitation of pre-RNA by Sof1p (Fig. 6), ruling out such an interdependence.

Two different lines of evidence indicate that Sof1p does interact with the generic box C/D protein Nop1p. One is the recent observation that the two proteins may be imported into the nucleus as a complex (22), and the other is their genetic interaction (16). From the data presented here, it can be concluded that, although they may interact during nuclear import, once they arrive in the nucleus, Sof1p and Nop1p lead separate lives until they meet again in the 90S preribosome. Exactly where this meeting takes place remains to be established, since the declassification of Sof1p as a U3-specific protein opens up numerous other possibilities.

Although Sof1p contains no RNA-binding motifs, we have considered a direct interaction of the protein with 35S pre-rRNA. In contrast to Rrp5p, however (34), we were unable to detect any RNA-binding activity of Sof1p in vitro, using synthetic pre-rRNA transcripts (data not shown). Therefore, loading of Sof1p onto the preribosome probably depends upon specific protein-protein interactions with one or more of the other components of the processome. Our data exclude Rrp5p, which, however, still leaves a host of other candidates to be tested.

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