RNA (2002), *8*:1348–1360. Cambridge University Press. Printed in the USA.
Copyright © 2002 RNA Society.
DOI: 10.1017/S1355838202027048

An active precursor in assembly of yeast nuclear ribonuclease P

CHATCHAWAN SRISAWAT,[1] FELICIA HOUSER-SCOTT,[1] EDOUARD BERTRAND,[2] SHAOHUA XIAO,[1] ROBERT H. SINGER,[3] and DAVID R. ENGELKE[1]

[1]Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-0606, USA
[2]Institut de Genetique Moleculaire de Montpellier, IFR 24-Centre National de la Recherche Scientifique UMR 5535, 34000 Montpellier, France
[3]Department of Anatomy and Structural Biology and Cell Biology, Albert Einstein College of Medicine, New York, New York 10461, USA

ABSTRACT

The RNA–protein subunit assembly of nuclear RNase P was investigated by specific isolation and characterization of the precursor and mature forms of RNase P using an RNA affinity ligand. Pre-RNase P was as active in pre-tRNA cleavage as mature RNase P, although it contained only seven of the nine proteins found in mature RNase P. Pop3p and Rpr2p were not required for maturation of the *RPR1* RNA subunit and virtually absent from pre-RNase P, implying that they are dispensable for pre-tRNA substrate recognition and cleavage. The RNase P subunit assembly is likely to occur in the nucleolus, where both precursor and mature forms of RNase P RNA are primarily localized. The results provide insight into assembly of nuclear RNase P, and suggest pre-tRNA substrate recognition is largely determined by the RNA subunit.

Keywords: RNase P; *RPR2*; *POP3*; *RPR1*

INTRODUCTION

Ribonuclease P (RNase P) is a ribonucleoprotein (RNP) enzyme responsible for endonucleolytic cleavage of the 5′ leader sequences of precursor tRNAs (pre-tRNA) during tRNA biogenesis to generate the mature 5′ ends of tRNAs (Frank & Pace, 1998; Xiao et al., 2002). It is an essential enzyme present in all domains of life (Bacteria, Archaea, and Eukarya). In most instances, the enzyme consists of a single RNA subunit forming a catalytic core with varying amounts of protein. In bacteria, the RNase P holoenzymes are relatively simple, consisting of only one RNA and one small protein subunit, whereas in eukaryotes, the holoenzymes are far more complex. For example, nuclear RNase P in *Saccharomyces cerevisiae* consists of one RNA subunit, *RPR1* RNA (Lee & Engelke, 1989; Lee et al., 1991b), and at least nine known protein subunits (Pop1p, Rpp1p, Pop3p, Pop4p, Pop5p, Pop6p, Pop7p, Pop8p, and Rpr2p; Lygerou et al., 1994; Chu et al., 1997; Dichtl & Tollervey, 1997; Stolc & Altman, 1997; Chamberlain et al., 1998; Stolc et al., 1998; reviewed in Houser-Scott et al., 2001; Xiao et al., 2002). Because of the higher complexity of the eukaryotic nuclear RNase P, how these subunits interact and assemble to form functional holoenzymes is not well understood.

In *S. cerevisiae*, there is evidence linking a defect in RNase P holoenzyme assembly to defective maturation of the *RPR1* RNA subunit. *RPR1* RNA is initially synthesized as a precursor form (pre-*RPR1*), which contains the 5′ leader and 3′ trailer sequences flanking the mature domains (mature *RPR1*). The 5′ leader sequence is 84 nt long, containing A-box and B-box internal promoters for RNA polymerase III (Lee et al., 1991a). The 3′ trailer in the primary transcript is 34 nt long, where it ends in a stretch of five U residues corresponding to an RNA polymerase III terminator (Lee et al., 1991a, 1991b; Tranguch & Engelke, 1993). Both flanking sequences are removed sometime during or after subunit assembly to generate mature *RPR1* (369 nt long). Usually, mature *RPR1* is the predominant form in the cell, accounting for approximately 80–90% of total *RPR1* RNA (Lee et al., 1991b; Pagan-Ramos et al., 1996a). However, under conditions that interfere with holoenzyme assembly, *RPR1* RNA fails to be processed from pre-*RPR1* to mature *RPR1*, resulting in a reduction or disappearance of the mature form. Depletion of the protein subunits, except Pop3p, results in a

Reprint requests to: David R. Engelke, Department of Biological Chemistry, University of Michigan, 3200 MSRB III, 1150 W. Medical Center Drive, Ann Arbor, Michigan 48109-0606, USA; e-mail: engelke@umich.edu.

reduction of mature *RPR1* and relative accumulation of pre-*RPR1* (Lygerou et al., 1994; Chu et al., 1997; Dichtl & Tollervey, 1997; Stolc & Altman, 1997; Chamberlain et al., 1998; Stolc et al., 1998). Moreover, mutations of *RPR1* RNA in a region involved in binding to protein subunits were found to exhibit a defect in *RPR1* RNA maturation (Ziehler et al., 2001), whereas mutations of the most highly conserved residues involved in substrate binding and catalysis do not affect the maturation (Pagan-Ramos et al., 1996a, 1996b).

These findings suggest a link between RNase P holoenzyme assembly and the maturation of *RPR1* RNA. Therefore isolation and characterization of the precursor of RNase P might lead to a better understanding of the assembly process. In this study we used an RNA ligand to facilitate the specific affinity isolation of the precursor and mature forms of RNase P (pre- and mature RNase P; Srisawat et al., 2001; Srisawat & Engelke, 2002). Pre-RNase P was found to be catalytically active in vitro, with two protein subunits, Pop3p and Rpr2p, conspicuously underrepresented in the holoenzyme. In situ localization of pre-RNase P also showed that, like mature RNase P, it resides primarily in the nucleolus, suggesting that the assembly of subunits occurs in this compartment.

RESULTS

Affinity tagging of the precursor and mature forms of *RPR1* RNA

Although separation of pre-RNase P from the more abundant, mature RNase P is feasible, it is a lengthy process requiring several steps of high-resolution chromatographic separation because both of them have very similar chromatographic properties (Lee & Engelke, 1989; Chamberlain et al., 1996). This suggests that pre-RNase P is at least partially assembled into an RNP complex before processing into mature RNase P. During this protracted purification, there could be a loss of enzyme subunits and activity, making the isolated enzyme less likely to reflect the form found inside cells. Recently, we developed RNA affinity tags to facilitate the isolation of RNAs and RNPs (Srisawat & Engelke, 2001, 2002; Srisawat et al., 2001). Previously, *RPR1* RNA was tagged with a minimal Sephadex-binding RNA ligand (Srisawat et al., 2001; Srisawat & Engelke, 2002). This allows a rapid and specific isolation of the tagged RNase P using Sephadex beads as an affinity matrix.

To specifically isolate pre-RNase P using the RNA affinity tag, the 5′ leader was chosen as the candidate site to incorporate the tag. This structure is present only in pre-*RPR1* before being excised and destroyed posttranscriptionally during maturation. Curiously, the 5′ leader (Fig. 1A), which contains a unique, disposable internal promoter for RNA polymerase III, has only been found in budding yeasts. The structure of the 5′ leader in *Saccharomyces cerevisiae* is not known and phylogenetic analysis of the leader sequences from closely-related *Saccharomyces* species does not predict a conserved structure (C. Srisawat, A. Tranguch, & D. Engelke, unpubl. observations). A secondary-structure prediction program (Mathews et al., 1999; Zuker et al., 1999) and preliminary RNA footprinting data (A.J. Tranguch & D.R. Engelke, unpubl. data) were used to provide structure hypotheses to suggest promising sites for inserting the RNA tags. Two sites were tested for whether a functional pre-*RPR1* was produced. In the first construct, leader A, the RNA ligand was inserted near the end of the hairpin (between nt −5 and −9), and in the other, leader B, it was tagged at the very end, between nt +1 and −1. Both leader-tagged *RPR1* constructs avoided the internal promoter region (positions −20 to −65; Lee et al., 1991a) and could complement the loss of the wild-type *RPR1*, showing normal growth phenotypes (Fig. 1B).

In a separate set of strains, the RNA affinity ligand was inserted into the terminal loop of the P3 hairpin domain of mature *RPR1* (Fig. 1A). This site is accessible in solution (Tranguch et al., 1994) and not essential for function (Lindahl et al., 2000). Because the P3 domain is also found in pre-*RPR1*, affinity isolation of the P3-tagged *RPR1* will result in a recovery of both pre- and mature RNase P. However, because mature *RPR1* is much more abundant, the holoenzyme isolated using this construct is primarily mature RNase P and was used as a control for pre-RNase P. The resulting P3-tagged *RPR1* construct could complement the wild-type *RPR1* and showed a normal growth phenotype (Fig. 1B).

Isolation of the precursor and mature forms of RNase P containing tagged *RPR1* RNA

To test whether RNase P containing the leader- or P3-tagged *RPR1* RNA can be specifically isolated using Sephadex, crude extracts were prepared from four different strains containing wild-type (as a control), leader-tagged (A or B), or P3-tagged *RPR1* as a sole source of *RPR1* RNA. The extracts were then incubated with Sephadex G-200 followed by elution with soluble dextran. The northern blot analysis of the starting cellular extracts is shown in Figure 2A. The precursor forms of leader-tagged *RPR1* (A and B) are expected to be 29 and 34 nt longer than the wild-type pre-*RPR1*, respectively, due to the inserted RNA tags. Their relative sizes in the northern blot are consistent with this. However, the mature forms of both leader-tagged *RPR1* constructs are of the same size as mature *RPR1*, as expected. In addition, ratios of pre-*RPR1* to mature *RPR1* are approximately normal, suggesting that the insertion of the RNA tag does not interfere with the removal of the 5′ leader in the leader-tagged *RPR1*. In the P3-tagged *RPR1*, both the precursor and mature forms are 35 nt longer than wild-type *RPR1*, as expected.

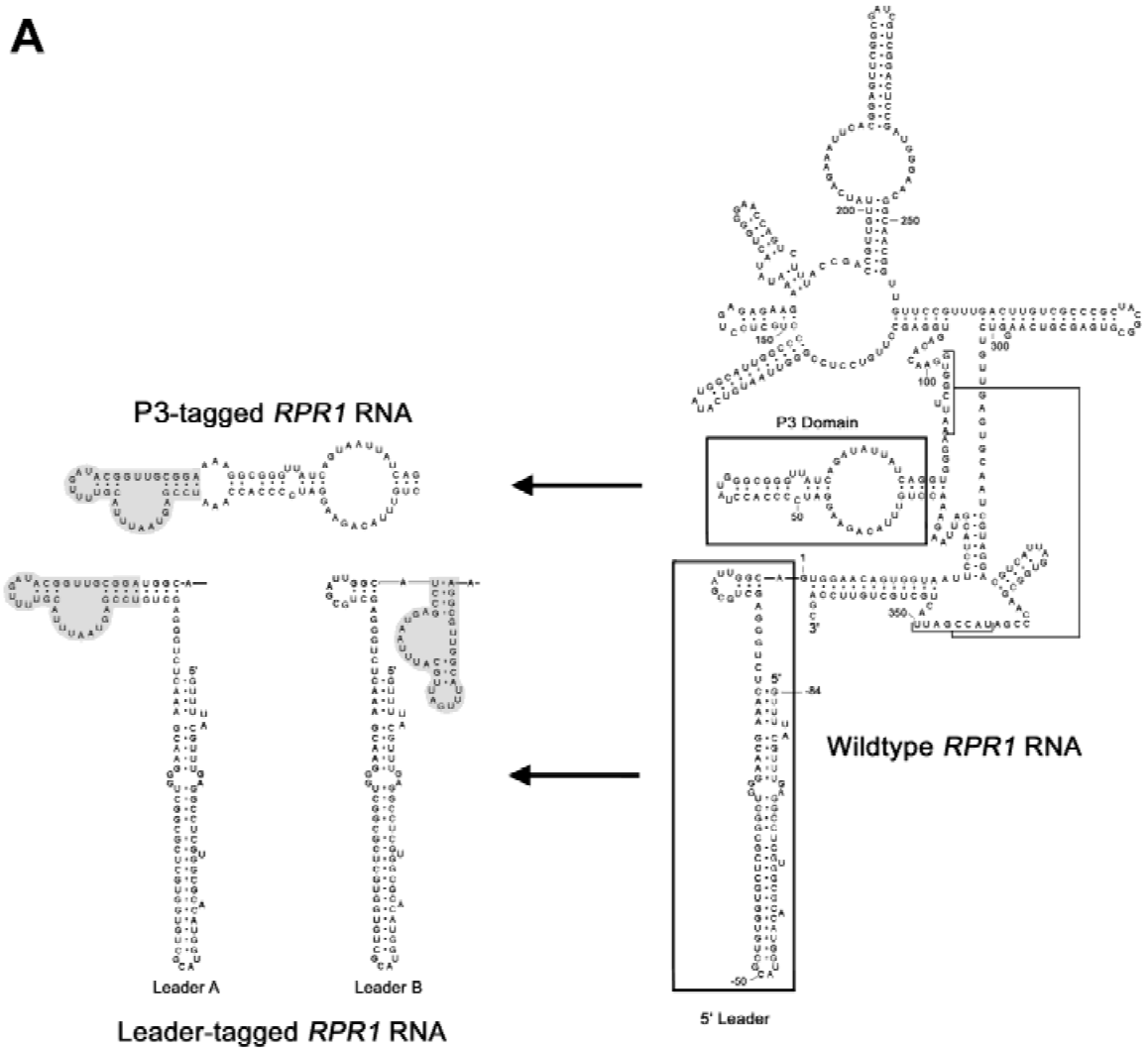

FIGURE 1. Affinity tagging of *RPR1* RNA with Sephadex-binding RNA ligand. **A:** The secondary structure of *RPR1* RNA according to Frank et al. (2000) is shown with the sequence of mature *RPR1* RNA starting at the nucleotide position +1. The predicted structures of the 5′ leader (from −1 to −84) and the P3 domain are shown in rectangles. The minimal binding motif of Sephadex-binding RNAs (in shaded areas) are inserted into the 5′ leader and the end of P3 domain to generate the leader-tagged (A and B) and P3-tagged *RPR1* RNA, respectively. **B:** Growth phenotypes of the yeast strains containing either wild-type, leader-tagged (A or B), or P3-tagged *RPR1* at various temperatures (15, 25, and 37 °C). The strains containing tagged *RPR1* have growth phenotypes indistinguishable from that of the wild-type strain.

The northern blot analysis of the eluates (Fig. 2A) shows that no detectable RNase P is pulled down with Sephadex from the crude extract containing untagged *RPR1*. For both leader-tagged *RPR1* strains, only the precursor forms are specifically recovered in the eluate. The mature forms, which have the tagged 5′ lead-

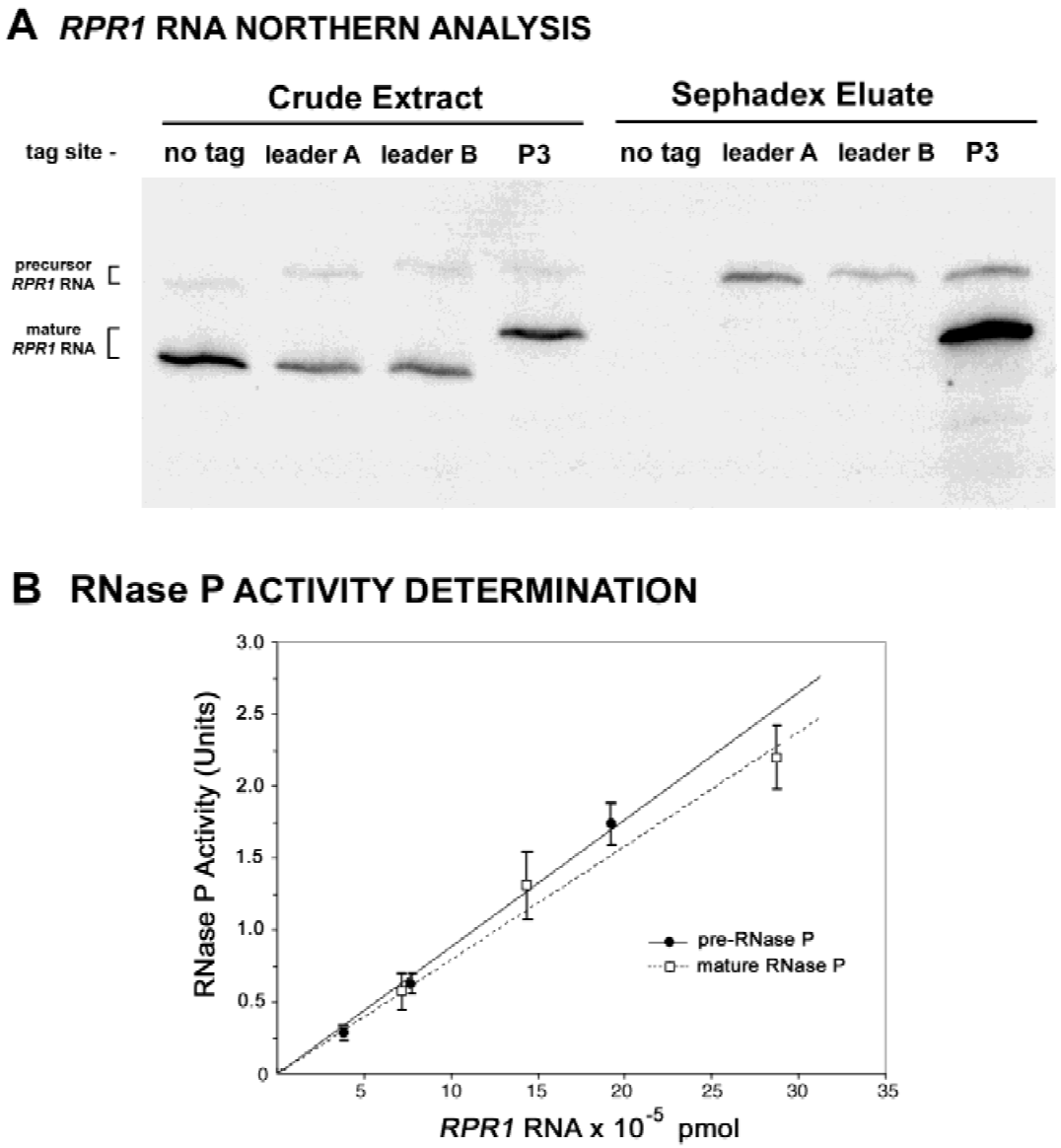

FIGURE 2. Isolation of RNase P containing the leader- or P3-tagged *RPR1* RNA. Crude extracts from the yeast strains containing wild-type (no tag), leader-tagged (A or B), or P3-tagged *RPR1* (P3/mature) were incubated with Sephadex G-200. After binding, the beads were washed and gently eluted with dextran. **A:** The presence of *RPR1* RNA in the crude extracts and eluates was analyzed by northern blot analysis. Exclusively, pre-*RPR1* was isolated with the leader-tagged constructs, whereas the P3-tagged constructs isolated mostly mature *RPR1* with low levels of pre-*RPR1*. **B:** RNase P activity in the leader-tagged versus P3-tagged RNase P eluates. Affinity isolates containing only the leader-tagged pre-RNase P (●), or the P3-tagged, mostly mature RNase P (□) were titrated in pre-tRNA cleavage assays. The RNase P activity is plotted against the amount of *RPR1* RNA subunit in the reaction. The RNase P-specific activities (units/picomole of *RPR1* RNA) were calculated from the slopes and are indistinguishable.

ers removed, do not bind to Sephadex. Using the P3-tagged *RPR1* strain, the eluate contains primarily the mature form, reflecting the relative abundance of precursor and mature forms in the cell. The results showed that the RNA affinity tags could be used successfully to facilitate specific isolation of RNase P containing tagged *RPR1* RNA. Because either leader A or leader B constructs worked well for isolation of the precursor, only the leader A construct was used for the remainder of the study.

The precursor of RNase P is enzymatically active in vitro

After affinity isolation with Sephadex, the RNase P activity in the eluates from wild-type and leader- or P3-tagged *RPR1* strains was assayed for the ability to cleave pre-tRNA substrates in vitro. Only the eluates from the strains containing the leader- and P3-tagged *RPR1* show enzymatic activity, suggesting that pre-RNase P is also catalytically active. To make the RNase

P activity from pre- and mature RNase P comparable by taking into account the amount of the holoenzyme recovered, the specific activity, defined as enzymatic units of RNase P per picomole of *RPR1* RNA, was determined (Fig. 2B). The results showed that both pre- and mature RNase P are approximately equally active, with a specific activity of 8×10^3 U/pmol of *RPR1* RNA for both forms. Unlike the RNase P RNA subunits in bacteria and some archaea that are enzymatically active without protein subunits (Guerrier-Takada & Altman, 1984, 1986; Pannucci et al., 1999), eukaryotic RNase P RNAs alone have never been shown to be catalytically active. The data suggested that most of pre-*RPR1* was not just naked RNA transcripts, but instead is assembled, partially or fully, with the protein subunits.

Pre-RNase P holoenzyme contains only seven of the nine essential protein subunits

Because pre-RNase P is enzymatically active, it is particularly interesting to analyze the protein subunit composition of pre-RNase P and compare it with that of mature RNase P. The protein subunits of highly purified nuclear RNase P consist of at least nine subunits that are essential for function in vivo: Pop1p, Rpp1p, Pop3p, Pop4p, Pop5p, Pop6p, Pop7p, Pop8p, and Rpr2p (Chamberlain et al., 1998). With the exception of Rpr2p, which is the unique subunit of RNase P, these proteins are also found as parts of a physically distinct, but closely related ribonucleoprotein enzyme, ribonuclease MRP (RNase MRP; Srisawat & Engelke, 2001). To study the RNase P subunit composition, we have generated nine different yeast strains, each of which has one protein subunit tagged with triple hemagglutinin (3×HA) epitopes. These strains have either the leader- or P3-tagged *RPR1* as a sole source of *RPR1* RNA so that either precursor or total nuclear RNase P can be specifically isolated. The associated protein subunits can be analyzed by western blot using an antihemagglutinin antibody.

Pre- and mature RNase P from the above strains expressing the 3×HA-tagged protein subunits were isolated using Sephadex G-200 as an affinity resin. The eluates were then split into two parts; one part was used for northern analysis of *RPR1* RNA, and the other was used for western analysis to detect the presence of a 3×HA-tagged protein subunit. The northern analysis of *RPR1* RNA (Fig. 3B) in the eluates from the strains containing the leader- or P3-tagged *RPR1* shows the expected recovery of either only pre-RNase P or mostly mature RNase P with low levels of precursor. Western blot analysis of the eluates with an antihemagglutinin antibody (Fig. 3A) shows that most of the protein subunits are associated with pre-RNase P at approximately the same levels as in mature RNase P, as judged from protein levels normalized to the amount of RNA subunit in the eluate (Fig. 3B). Pop1p, Pop4p, Pop5p, Pop6p, Pop7p, Pop8p, and Rpp1p are present at similar levels, although the Pop7p levels in the precursor and mature forms could not be quantitatively determined with as much confidence because of consistently low recoveries and low signal strength in the western blots. In contrast, Pop3p and Rpr2p in the pre-RNase P are reduced at least 80–90% compared to the levels in the mature form.

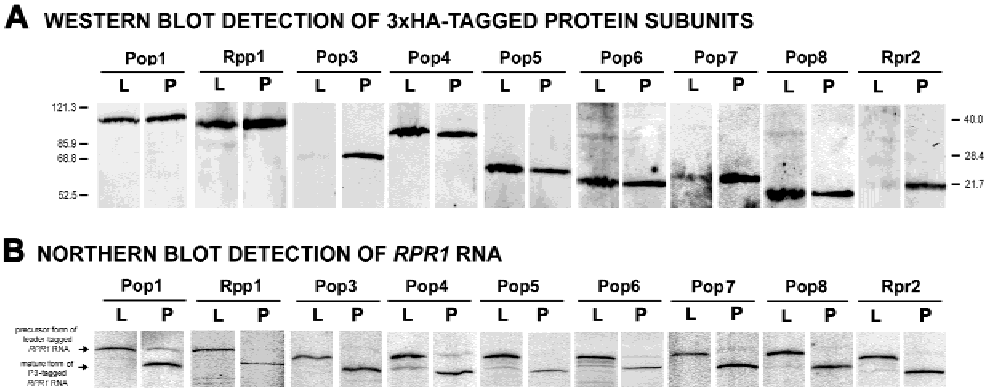

FIGURE 3. The protein subunit composition of pre-RNase P and mature RNase P. Crude extracts from yeast strains containing 3×HA-tagged protein subunits and either the leader- or P3-tagged *RPR1* were incubated with Sephadex, after which the beads were washed and eluted with dextran. The eluates were analyzed for the presence of protein subunits and *RPR1* RNA by western (**A**) and northern blot analysis (**B**), respectively. Pre-*RPR1* and mostly mature *RPR1* are specifically isolated, respectively, from the strains containing the leader-tagged (L) or P3-tagged *RPR1* (P) constructs as shown in **B**. (The small-sized bands found in the eluates of some leader-tagged *RPR1* strains are not the correct sizes for mature *RPR1* and are likely to be partial degradation products of pre-*RPR1* that occur during isolation.) From the western analysis (**A**), the protein subunit composition of pre-RNase P is similar to that of mature RNase P, except that Pop3p and Rpr2p are nearly absent. The protein samples were analyzed on either 7.5% (Pop1p) or 12% SDS-PAGE (the other protein subunits). The molecular weight markers for both gels are shown at the left and right in **A**. Some protein subunits, that is, Rpp1p and Pop7p, migrate as proteins with molecular weight larger than predicted by sequence.

Depletion of Pop3p or Rpr2p does not strongly affect the maturation of *RPR1* RNA

These studies suggest that Pop3p and Rpr2p enter the RNP complex late in the assembly process, either just before or after cleavage of the 5′ leader in pre-*RPR1*. It is possible that addition of one or both subunits to pre-RNase P is a needed signal for rapid cleavage of pre-*RPR1* to mature *RPR1*. To test whether these two protein subunits are required for *RPR1* RNA maturation, they were selectively depleted and the effects on the maturation were examined. The chromosomal copies of Pop3p and Rpr2p were tagged at the N-termini with the 3×HA epitopes (in two separate strains) and the genes were placed under control of *GAL1* promoters. Parallel depletion studies were also done with Pop1p as a control, as loss of functional Pop1p is known to disrupt *RPR1* RNA maturation (Lygerou et al., 1994). All strains expressed the P3-tagged *RPR1* to facilitate isolation of the holoenzyme. The strains were grown in galactose-containing media (YPGR) before shifting to media with dextrose (YPD) for 12–18 h to turn off the indicated subunit expression, resulting in depletion of the protein subunits. RNase P was isolated and examined for the presence of the 3×HA-tagged proteins and *RPR1* RNA. The western blot analysis (Fig. 4A) shows that both Pop3p and Rpr2p in the holoenzyme complexes are reduced to undetectable levels after 18 h in dextrose media, confirming that the expression of the proteins are suppressed and the existing protein pools are largely depleted. However, the ratios of pre-*RPR1* to mature *RPR1* are not strongly affected by Pop3p and Rpr2p depletion, as shown from the northern blot analysis in Figure 4B. *RPR1* RNA is able to be processed into mature *RPR1* when Pop3p and Rpr2p are depleted. In contrast, depletion of Pop1p after 12 h in YPD (longer depletion is lethal), which reduces Pop1p in RNase P holoenzymes by more than 90%, causes a severe defect in *RPR1* RNA maturation, resulting in an increased ratio of pre-*RPR1* over mature *RPR1* (Fig. 4).

A defect in maturation of *RPR1* RNA caused by Pop1p depletion is similar to that found in depletion studies of other subunits that are present at approximately the same abundance in both pre- and mature RNase P, that is, Rpp1p, Pop4p, Pop5p, Pop6p, Pop7p, and Pop8p (Chu et al., 1997; Dichtl & Tollervey, 1997; Stolc & Altman, 1997; Chamberlain et al., 1998; Stolc et al., 1998). This finding suggests that Pop3p and Rpr2p, which are largely absent from isolated pre-RNase P, are also not required for processing of pre-*RPR1* into mature *RPR1*.

The precursor of RNase P localizes in the nucleolus

We previously showed that, in *S. cerevisiae*, RNase P RNA and various pre-tRNAs were found mainly in the nucleolus, with additional nucleoplasmic foci (Bertrand et al., 1998). The question arises whether pre-*RPR1* is assembled outside the nucleolus and imported into the nucleolus as the mature enzyme. To determine the localization of pre-RNase P, a fluorescent oligonucleotide probe specific to the 5′ leader sequence was used for in situ hybridization to fixed yeast cells. As shown in Figure 5, pre-RNase P seems to reside predominantly in the nucleolus. Together with the previous demonstration of nucleolar location of the bulk of RNase P, this suggests that the nucleolus might be the site for assembly and maturation of nuclear RNase P.

DISCUSSION

A precursor form of *RPR1* RNA containing a long leader sequence is a unique feature of RNase P RNA subunits from *Saccharomyces* species. The 5′ leader contains the internal A- and B-box elements typical of tRNA-class RNA polymerase III promoters (Lee et al., 1991a, 1991b). The fact that the leader structure is removed and degraded (Lee et al., 1991a, 1991b) pro-

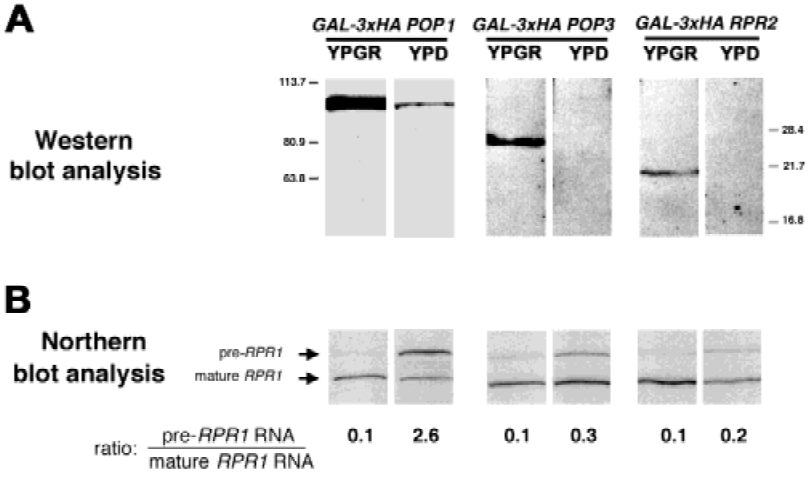

FIGURE 4. Depletion of Pop3p and Rpr2p does not affect the maturation of *RPR1* RNA. Yeast strains containing the P3-tagged *RPR1* and 3×HA-tagged Pop1p, Pop3p, or Rpr2p were generated. The proteins were tagged at the N-termini and their expression was regulated under *GAL1* promoters. Crude extracts were prepared from the cells grown in galactose-containing media (YPGR) or after 12–18 h in dextrose-containing media (YPD) to shut down the corresponding subunit expression. RNase P was isolated using Sephadex and the eluates were analyzed for the presence of protein subunits and *RPR1* RNA by western and northern blot analysis (**A** and **B**, respectively). After 18 h in YPD, the Pop3p and Rpr2p proteins in holoenzymes are decreased to undetectable levels. However, little effect is seen on the maturation of *RPR1* RNA with these depletions. In contrast, Pop1p depletion strongly affects the maturation, resulting in the accumulation of pre-*RPR1* and a decrease of mature *RPR1*, as indicated from the ratio of pre-*RPR1* over mature *RPR1*.

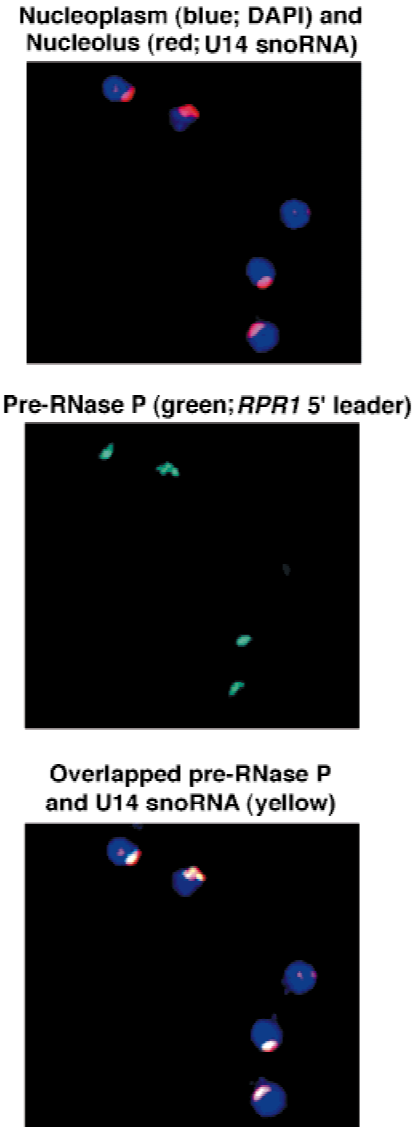

FIGURE 5. Pre-RNase P localizes primarily in the nucleolus in *S. cerevisiae*. Fluorescent in situ hybridization was used to study the localization of pre-RNase P. The top panel shows the nucleoplasm and nucleoli as visualized by DAPI staining (blue) and anti-U14 small nucleolar RNA probe (red). The middle panel shows the staining of pre-*RPR1* using the probe complementary to the 5′ leader of *RPR1* RNA. Both panels are merged together with overlap between the red and green signal in yellow as shown in the bottom panel.

vides the only established case of an internal promoter that is disposable after transcription. The enzyme that removes the 5′ leader has not yet been identified, but maturation does not appear to result from RNase P cleavage (C. Srisawat, unpubl. observations). Moreover, very little is known about the role of the 5′ leader in pre-RNase P or the reason for removing this structure from the mature form. The data from this study show that its presence does not significantly interfere with pre-tRNA cleavage in vitro.

The protein subunit composition of pre-RNase P was determined and compared with that of mature RNase P, which is known to have at least nine protein subunits (Pop1p, Rpp1p, Pop3p, Pop4p, Pop5p, Pop6p, Pop7p, Pop8p, and Rpr2p), all of which are essential and required for efficient RNase P function in vivo. The results presented here show that pre-RNase P has acquired most of the protein subunits contained in mature RNase P, except that Pop3p and Rpr2p are severely underrepresented relative to the other subunits. This further suggested that, unlike the other protein subunits, Pop3p and Rpr2p might not be required for maturation of *RPR1* RNA. We went on to show that depletion of either Pop3p or Rpr2p still allowed pre-*RPR1* to be processed into mature *RPR1*. This is in agreement with a previous study of Pop3p (Dichtl & Tollervey, 1997), although Rpr2p depletion for longer periods previously showed a partial maturation defect (Chamberlain et al., 1998). These data also suggest that neither subunit is critical for structural integrity of the holoenzyme.

Based on the current findings, a model of RNase P subunit assembly is proposed (Fig. 6). According to the model, the protein subunits that are required for *RPR1* RNA maturation (Pop1p, Rpp1p, Pop4p, Pop5p, Pop6p, Pop7p, and Pop8p) assemble with pre-*RPR1* first, either individually or as a preformed complex. Recent findings from yeast two-hybrid assays showed that RNase P protein subunits either from *S. cerevisiae* or human cells can extensively interact with each other in the absence of the RNA subunit (Jiang & Altman, 2001; Houser-Scott et al., 2002), suggesting that they might be able to associate together into a preformed complex. The protein contacts shown in Figure 6 reflect these established protein–protein interactions for the yeast subunits. The formation of such a complex might be useful for transporting some subunits to the nucleus and subnuclear compartments. For example, in human RNase P, hPop5 (a homolog of yeast Pop5p) and Rpp14 (no known yeast homolog) lack nuclear localization signals and are believed to be transported into the nucleus by piggyback mechanisms with other proteins (van Eenennaam et al., 2001a, 2001b; Jarrous, 2002).

The finding that pre-RNase P is active against pre-tRNA substrates in the virtual absence of Pop3p and Rpr2p is interesting in relation to previous studies of human RNase P subunits. Previously, no homolog of Pop3p has been identified in humans, whereas human Rpp21 is a known homolog of Rpr2p (Jarrous et al., 2001). However, one of the human subunits, Rpp38, can be stripped from the holoenzyme during Mono S chromatography without complete inactivation of RNase P activity (Eder et al., 1997; Jarrous et al., 1998; Jarrous & Altman, 2001). This would be consistent with our current results showing that Pop3p-deficient RNase P is catalytically active in vitro, if Rpp38 fulfills a role similar to Pop3p. Both Pop3p and Rpp38 are highly basic proteins (pI of 9.9 and 9.6, respectively) although the masses vary substantially (32 vs. 23 kDa). Align-

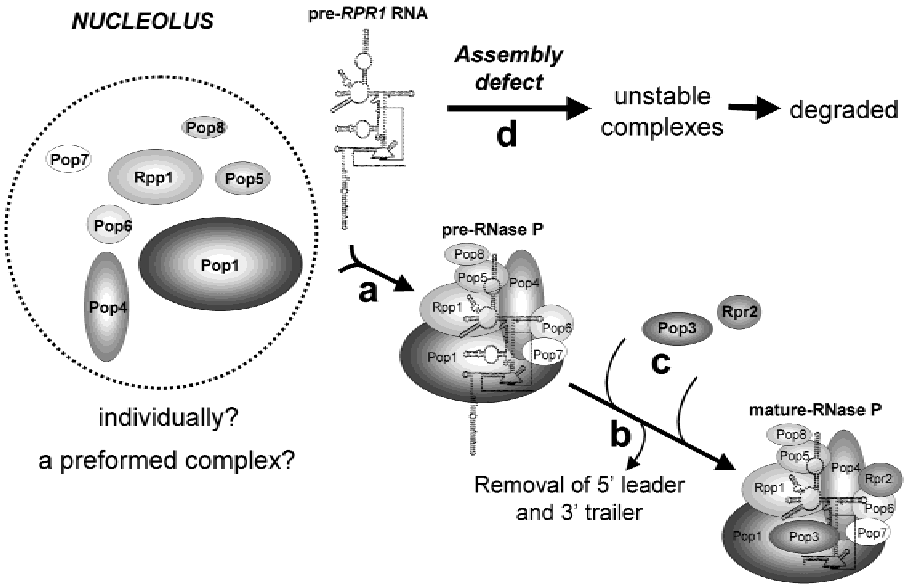

FIGURE 6. A hypothetical model of RNase P subunit assembly. In the nucleolus, pre-*RPR1* and all the protein subunits except Pop3p and Rpr2p are assembled first to form pre-RNase P complexes (**a**). It is not known whether the protein subunits associate with pre-*RPR1* individually or assemble together in a preformed complex before binding to pre-*RPR1*. Once the complexes are formed, pre-*RPR1* can be processed into mature *RPR1* without the requirement of Pop3p and Rpr2p association (**b**). Pop3p and Rpr2p are believed to assemble into the holoenzymes after the pre-RNase P complexes are formed, either before or after the processing of *RPR1* RNA (**c**), thus explaining the low abundance of these two subunits in pre-RNase P. In case of subunit assembly defects, the precursor complexes appear to be degraded, as the mature form eventually disappears, but there is no corresponding buildup in the pre-*RPR1* level (**d;** Chamberlain et al., 1998). The protein subunit organization used in this model is taken from Houser-Scott et al. (2002).

ment of these proteins and their putative homologs from mouse, cow, frog, zebra fish, and other yeasts (Fig. 7) shows significant similarity. We propose that Pop3p and Rpp38 are likely to be distantly related orthologs, and that neither is essential for pre-tRNA cleavage in vitro.

Previous studies of the isolated yeast Pop3p and human Rpp21 (Rpr2p homolog) show that they preferentially bind pre-tRNA substrates in vitro in the absence of other RNase P subunits, suggesting roles of these proteins in substrate binding and recognition (Brusca et al., 2001; Jarrous et al., 2001). However, these conclusions seem inconsistent with our current finding that RNase P deficient in either Pop3p or Rpr2p is catalytically active in vitro. Although these proteins might play some roles in binding pre-tRNAs or other RNAs in the cell, they are clearly not essential for recognition and cleavage of pre-tRNAs in vitro. It is therefore possible that the physiological ligands for these proteins are not pre-tRNAs, and the previous in vitro results reflect the difficulty in differentiating specific from nonspecific (but tight) binding by these basic proteins in vitro.

The activity of pre-*RPR1* complexes makes a particularly interesting point concerning the possible site of substrate recognition in RNase P. Nuclear RNase P shares eight protein subunits with RNase MRP, which is nucleolar and cleaves pre-ribosomal RNAs. The two known differences between the enzymes are the divergence of their RNA subunits and the existence of Snm1p in RNase MRP (Schmitt & Clayton, 1994), instead of the Rpr2p subunit found in RNase P (Chamberlain et al., 1998). It is reasonable to infer that one of these differences might be primarily responsible for the change in substrate specificity of the two enzymes. Because Rpr2p seems dispensable for pre-tRNA cleavage in vitro, we propose that the divergence of the RNA subunit is primarily responsible for the shift from pre-tRNA to pre-rRNA specificity.

In this study, the localization of pre-RNase P was found to be mainly in the nucleolus, consistent with a previous finding using probes to both mature *RPR1* and pre-tRNA (Bertrand et al., 1998). These combined results suggest that the assembly of RNase P might occur in the nucleolus, as has previously been proposed for human RNase P (Jacobson et al., 1997). However, in contrast to the localization of *RPR1* RNA in *S. cerevisiae*, the RNA subunit of human RNase P, H1 RNA, was found mainly in the nucleoplasm (Jarrous et al., 1998; Jarrous & Altman, 2001). When H1 RNA is microinjected into the nucleus, it migrates to the nucleolus and transiently localizes in that compartment before migrating into the nucleoplasm (Jacobson et al., 1997), suggesting that the nucleolus might be the site for holoenzyme assembly before it migrates to other compartments to perform its functions. This notion agrees well with the localization of human RNase P protein subunits: hPop1, hPop5, Rpp14, Rpp29 (homolog of Pop4p), Rpp30 (homolog of Rpp1p), and Rpp38

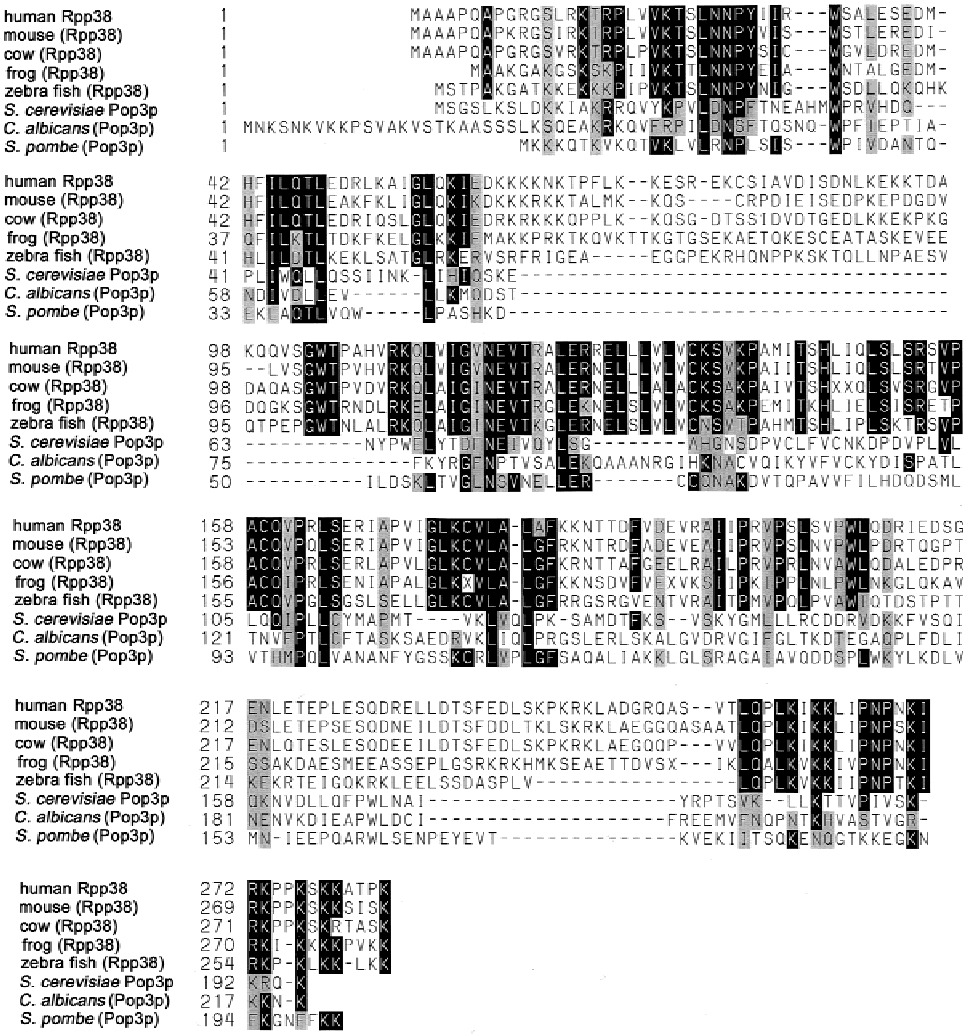

FIGURE 7. Amino acid sequence alignment of *S. cerevisiae* Pop3p, human Rpp38, and the putative homologs. The putative homologs of human Rpp38 in mouse (*Mus musculus*), cow (*Bos taurus*), frog (*Xenopus laevis*), and zebra fish (*Danio rerio*) were derived from expressed sequence tag databases. The sequences of *Schizosaccharomyces pombe* and *Candida albicans* are putative homologs of Pop3p. The residues that are conserved in identity (five out of eight sequences) or similarity (seven out of eight sequences) are marked in black or gray boxes, respectively.

(Lygerou et al., 1996; Jarrous et al., 1999; van Eenennaam et al., 2001a; Jarrous, 2002). However, these proteins are also likely subunits of the related ribosomal RNA processing enzyme, RNase MRP (Lygerou et al., 1996; Pluk et al., 1999; van Eenennaam et al., 1999, 2000), so that it is difficult to tell in these human studies whether the nucleolar fluorescent signal originates from proteins that are associated with RNase P, with RNase MRP, or with some other complex. In any case, the location of most of the protein subunits in the nucleolus makes it a potential site for holoenzyme assembly. It should be noted that Rpp21, a homolog of Rpr2p, is the only human RNase P protein subunit so far that is found primarily in the nucleoplasm (Jarrous et al., 2001). Thus, there may be differences in the localization of mature RNase P between yeast and human.

Given that the pre-RNase P complex is active, the question arises whether it might have independent functions in the cell, in addition to being a precursor of the mature form of RNase P. Bacterial RNase P has several types of substrates other than pre-tRNAs, including pre-4.5S RNA, pre-10Sa RNA (tmRNA) in *Escherichia coli*, and a polycistronic mRNA (Peck-Miller & Altman, 1991; Alifano et al., 1994; Komine et al., 1994). At first glance, it does not seem likely that *S. cerevisiae* pre-RNase P could fulfill any universally necessary function, as only budding yeasts are known to have this sort of long, promoter-containing precursor of RNase P RNA. However, other eukaryotes might have similar variants of the RNase P holoenzyme lacking Pop3p and Rpr2p equivalents, even though they lack the long leader on the RNA subunit. Although no alternative substrates have yet been identified for nuclear RNase P, it is possible that the "pre-RNase P" activity participates in cellular pathways that are distinct from the roles played by the "mature RNase P."

MATERIALS AND METHODS

Constructing the leader- and P3-tagged *RPR1*

The minimal motif of D8 Sephadex-binding aptamer (33 nt long; Srisawat et al., 2001; Srisawat & Engelke, 2002) was inserted into *RPR1* using overlap-extension PCR (Ling & Robinson, 1997). The structures of *RPR1* and its tagged constructs were generated using RNAviz program (De Rijk & De Wachter, 1997). The PCR products containing wild-type *RPR1* (positions −382 to +465) tagged with Sephadex-binding RNA ligands were cut with *Sac*II and *Apa*I and cloned into the *Sac*II-*Apa*I sites of pRS315. The *RPR1* gene used in this study contains an engineered *Nde*I site between +132 to +141 for a convenient cloning site and it does not affect function.

Yeast strains

FSY1 strain was generated from *S. cerevisiae* W3031A (*MAT*a *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*). The *RPR1* gene in FSY1 was disrupted by *kan*^{r} (between nt 44–343 of mature *RPR1*) using PCR-based gene deletion (Longtine et al., 1998), and *RPR1* RNA was derived from YCp50-*RPR1* plasmid (Lee et al., 1991b). The strain was transformed with pRS315 containing wild-type *RPR1*, leader-tagged *RPR1*, or P3-tagged *RPR1*. YCp50-*RPR1* was later removed using 5-fluoroorotic acid (5-FOA).

Yeast strains containing 3×HA-tagged protein subunits of RNase P were generated from W3031A. The chromosomal copies of the genes were tagged at the C-termini with triple hemagglutinin epitopes (with *kan*^{r} as a marker) using PCR-mediated gene modification techniques (Longtine et al., 1998) so that each tagged protein was the sole source in the cell and expressed under its endogenous promoter. *RPR1* genes in these strains were also disrupted in the same fashion as in FSY1 by *HIS3*, with *RPR1* RNA expressed from YCp50-*RPR1* plasmid. The C-terminal 3×HA-tagged Pop1p, Pop3p, Pop5p, Pop6p, Pop8p, and Rpr2p showed normal growth phenotypes, whereas the tagged Rpp1p, Pop4p, and Pop7p grew somewhat slowly; their doubling times were about twice as long as that of the wild type. However, we found that the C-terminal 3×HA-tagged Pop1p and Pop7p could not be used in this study and needed to be reconstructed. The 3×HA-tagged Pop1p could not be detected by western analysis, possibly because the 3×HA epitopes at the C-terminus might be cleaved off in vivo or during purification. This proteolytic degradation of Pop1p at the C-terminus had been previously observed during the purification of RNase P (Chamberlain et al., 1998). For Pop7p, the strain containing the C-terminal tagged protein was nonviable in combination with the leader-tagged *RPR1*, but not the P3-tagged *RPR1*. Therefore, the yeast strains containing Pop1p or Pop7p tagged with 3×HA at the N-terminus were generated as described below.

The strain containing 3×HA-tagged Pop1p was derived from W3031A with *POP1* gene disrupted with the *natI* gene (Goldstein & McCusker, 1999) using PCR-mediated gene deletion, and Pop1p was supplied from p413GAL-*3×HA-POP1*. This plasmid was generated by cloning PCR fragments containing a full-length *POP1* (nucleotide positions +1 to +2628) into the *Xma*I-*Sal*I site between the *GAL1* promoter and *CYC1* terminator of p413GAL (a gift of D.J. Thiele, University of Michigan). The 3×HA epitopes were later tagged into the N-terminus of *POP1* by PCR mutagenesis. Its *RPR1* gene was disrupted with *kan*^{r} as described in FSY1 with *RPR1* RNA expressed from YCp50-*RPR1*.

The strains containing the N-terminal 3×HA-tagged Pop3p, Pop7p, and Rpr2p were generated from JLY1 strain (Lee et al., 1991b), which is W3031A with *RPR1* gene disrupted by *HIS3* and *RPR1* RNA is derived from YCp50-*RPR1* plasmid. The chromosomal copies of the above genes in JLY1 were tagged at the N-termini with *GAL1* promoter-driven 3×HA epitopes (with *kan*^{r} as a marker) using PCR-mediated gene modification techniques (Longtine et al., 1998).

Each 3×HA-tagged subunit strain was then transformed with either pRS315 containing leader- or P3-tagged *RPR1*, and YCp50-*RPR1* was later cured using 5-FOA.

Isolation of the leader- and P3-tagged RNase P using Sephadex G-200

The FSY1 strains containing pRS315 expressing wild-type, leader-tagged (A or B), or P3-tagged *RPR1* were grown in 1 L of YPD at 30 °C until OD ~1–2. The cells were harvested and washed three to four times with distilled water (~10 pellet volumes). The pellets were resuspended in 3 mL of lysis buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 0.1% Triton-X 100, and 10% glycerol) containing protease inhibitors Complete® (Roche). The cells were lysed by vortexing with 10-15 g of acid-washed glass beads, size 425–600 μM (Sigma) for 20–30 min. Crude extracts were centrifuged at 14,000 × *g* for 5 min at 4 °C to remove particulate matter, and the protein concentrations were determined using the Micro Bicinchoninic acid Assays (Pierce).

The Sephadex G-200 beads used in affinity isolation were prepared as follows: 1 g of Sephadex G-200-120, particle diameter 40–120 μM (Sigma), were swollen at room temperature overnight in 50–100 mL of buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, and 100 mM NaCl). The swollen beads were washed several times with lysis buffer and the 50% suspension of Sephadex beads in lysis buffer was made.

Crude extracts containing 25 mg of protein were incubated with 150 μL of Sephadex G-200 beads for 1 h at 4 °C. After the binding step, the beads were washed with 1 mL of lysis buffer five times, for 3 min each. They were then transferred into Ultrafree-MC centrifugal units, 0.45 μM size (Millipore), and washed twice briefly with 350 μL of lysis buffer. Complexes were eluted from the beads by incubating with 300 μL of 50 mg/mL dextran, M_r ~ 6,000 (Fluka) in lysis buffer containing protease inhibitors for 30 min at 4 °C. The eluates were used for northern blot analysis and RNase P activity assays.

Northern blot analysis of *RPR1* RNA

The RNA was extracted with Trizol reagents (Invitrogen) as described by the manufacturer. It was then loaded into 5% denaturing polyacrylamide gels in 8 M urea and electroblotted to a Nytran-Supercharge membrane (Schleicher & Schuell). The blot was hybridized with 1–2 × 10^7 cpm of ^{32}P-radiolabeled antisense *RPR1* RNA probe complemen-

tary to positions +80 to +369 at 55 °C overnight, washed, and visualized using a PhosphorImager. The band intensity was quantified using IPLab Gel analysis software (Signal Analytics).

RNase P activity assay

The assays were done by incubating samples with ^{32}P-radiolabeled pre-tRNA$^{Asp\text{-}AU}$ substrates (Hollingsworth & Martin, 1987), 5,000 cpm (25 pmol) at 37 °C for 15 min. The reactions were done in 5 μL lysis buffer and the products were separated by electrophoresis through 10% denaturing polyacrylamide gels in 8 M urea. The radioactive intensity of substrate and product bands was quantitated to determine the percentage of substrate cleaved by RNase P. The specific activity was defined as units of RNase P activity per pmol of *RPR1* RNA. One unit of RNase P activity is arbitrarily defined as an amount of enzyme that can cleave 20% of 25 pmol of pre-tRNA substrates in 15 min at 37 °C. The amount of *RPR1* RNA in the samples was determined from northern blot analysis with a known amount of in vitro-transcribed *RPR1* RNA standard. The assays were done twice and in triplicate. The RNase P activity (mean ± standard error) is plotted against the amount of *RPR1* RNA subunit in the reaction as shown in Figure 2B and the specific activities were determined from the slopes.

Protein subunit determination

Yeast strains containing 3×HA-tagged subunits and either the P3- or leader-tagged *RPR1* were grown in 0.4 L or 4 L, respectively, of the appropriate media (either YPD or YPGR). The cells were harvested and washed well three to four times with distilled water (~10 × pellet volumes). The cells were lysed using glass beads as described above. For strains containing the P3-tagged *RPR1*, 25 mg protein of crude extracts were incubated with 250 μL of Sephadex beads at 4 °C for 1 h. After the binding step, the beads were washed with 1 mL of lysis buffer five times, for 3 min each. They were then transferred into Ultrafree-MC centrifugal units, 0.45 μM size (Millipore), and washed twice briefly with 350 μL of lysis buffer. For strains containing the leader-tagged *RPR1*, ~200–250 mg protein of crude extracts were incubated with 1,000 μL of Sephadex beads for 1 h at 4 °C. The beads were washed three times, 5 min each, with 12.5 mL of lysis buffer. The beads were transferred to Ultrafree-CL centrifugal units, 0.45 μM size (Millipore), and washed once briefly with 1.5 mL of lysis buffer.

The elution step was done by incubating the beads with 0.5 or 2 mL of soluble dextran in lysis buffer for 30 min at 4 °C for the P3- or leader-tagged *RPR1* strains, respectively. The dextran used in this elution step was either 50 mg/mL dextran, M_r ~ 6,000 (Fluka) or 100 mg/mL enzymatically synthesized dextran, M_r ~ 1,500 (Fluka), in lysis buffer containing protease inhibitors. The latter was preferred because of its smaller size, which made removal easier. The eluates were subsequently concentrated in Microcon YM-50 (50,000 D molecular weight cutoff) until the total volume was reduced to ~50 μL. They were then split into two portions; the first one (~10–20% of the eluates) was used for northern blot analysis for *RPR1* as described above, and the other used for western blot analysis for 3×HA-tagged proteins.

Western blot analyses were done in 7.5% and 12% SDS-PAGE for Pop1p and the other protein subunits, respectively. Proteins were then transferred to Polyscreen® PVDF membranes (NEN). The blots were incubated with antihemagglutinin monoclonal antibody HA.11 (Covance) followed by goat anti-mouse IgG polyclonal antibodies conjugated with horseradish peroxidase (Chemicon). The signals were developed using an enhanced chemiluminescent substrate, SuperSignal WestPico Chemiluminescent (Pierce), and exposed to Biomax-ML films (Kodak). The films were scanned and the band density was quantitated using IPLab Gel analysis software (Signal Analytics) and normalized to the amounts of *RPR1* RNA in the same eluates in order to compare the amounts of the protein subunits in pre- and mature RNase P.

Depletion of Pop1p, Pop3p, and Rpr2p

The yeast strains containing the N-terminal 3×HA-tagged Pop1p, Pop3p, or Rpr2p under *GAL1* promoters were grown in 0.5 L of YPGR media at 30 °C overnight (final OD_{600} ~ 2.5–3). The cells were harvested and used to inoculate into 1 L of YPD to a final OD_{600} of ~0.1. The cells were further grown at 30 °C for 12 h for Pop1p or 18 h for Pop3p and Rpr2p before being harvested. Crude extracts from cells grown in YPGR and YPD were prepared and used for affinity isolation with Sephadex beads as described above. The eluates were analyzed for the presence of proteins and *RPR1* RNA.

Fluorescent in situ hybridization

The haploid yeast strain W3031A was used for the localization study of pre-RNase P. The detailed procedures were described previously (Bertrand et al., 1998). The antisense pre-*RPR1* RNA probe, annealing to nt −1 to −48 of the 5′ leader sequence was labeled at the 5′ end with fluorescein and had the sequence 5′-TGCCAATCGCAGCTCCCAGA GTTTCGTTCCCAGCCGCGAGCACCACAG-3′. The CY3-labeled antisense RNA probe for U14 snoRNA was prepared as described (Bertrand et al., 1998).

Sequence alignment

Expressed sequence tag database searches were performed using BLAST 2.0. The accession numbers of Rpp38, Pop3p, and their putative homologs are as follows; human Rpp38 (P78345), mouse *Mus musculus* (Mm18998), cow *Bos taurus* (BF603940, AV592481), frog *Xenopus laevis* (BJ096419, BJ100606), zebra fish *Daniro rerio* (BI888555, BM036050, Dr_2574_1), *S. cerevisiae* Pop3p (NP014117), *Schizosaccharomyces pombe* (SPCC16C4.05), and *Candida albicans* (CA6042). The sequence alignment was performed using CLUSTAL W (Thompson et al., 1994) and MULTALIN (Corpet, 1988) followed by manual alignment.

ACKNOWLEDGMENTS

We thank May Tsoi for her invaluable technical help in preparing growth media. C.S. was supported by scholarships from Royal Thai Government, Siriraj Hospital Mahidol University, and Horace H. Rackham School of Graduate Studies at the University of Michigan. This work was supported

by grants from the National Institutes of Health to D.R.E (GM34869) and R.H.S. (GM54887 and GM57071).

Received July 11, 2002; returned for revision July 25, 2002; revised manuscript received July 31, 2002

REFERENCES

Alifano P, Rivellini F, Piscitelli C, Arraiano CM, Bruni CB, Carlomagno MS. 1994. Ribonuclease E provides substrates for ribonuclease P-dependent processing of a polycistronic mRNA. *Genes & Dev 8*:3021–3031.

Bertrand E, Houser-Scott F, Kendall A, Singer RH, Engelke DR. 1998. Nucleolar localization of early tRNA processing. *Genes & Dev 12*:2463–2468.

Brusca EM, True HL, Celander DW. 2001. Novel RNA-binding properties of Pop3p support a role for eukaryotic RNase P protein subunits in substrate recognition. *J Biol Chem 276*:42543–42548.

Chamberlain JR, Lee Y, Lane WS, Engelke DR. 1998. Purification and characterization of the nuclear RNase P holoenzyme complex reveals extensive subunit overlap with RNase MRP. *Genes & Dev 12*:1678–1690.

Chamberlain JR, Pagan R, Kindelberger DW, Engelke DR. 1996. An RNase P RNA subunit mutation affects ribosomal RNA processing. *Nucleic Acids Res 24*:3158–3166.

Chu S, Zengel JM, Lindahl L. 1997. A novel protein shared by RNase MRP and RNase P. *RNA 3*:382–391.

Corpet F. 1988. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res 16*:10881–10890.

De Rijk P, De Wachter R. 1997. RnaViz, a program for the visualisation of RNA secondary structure. *Nucleic Acids Res 25*:4679–4684.

Dichtl B, Tollervey D. 1997. Pop3p is essential for the activity of the RNase MRP and RNase P ribonucleoproteins in vivo. *EMBO J 16*:417–429.

Eder PS, Kekuda R, Stolc V, Altman S. 1997. Characterization of two scleroderma autoimmune antigens that copurify with human ribonuclease P. *Proc Natl Acad Sci USA 94*:1101–1106.

Frank DN, Adamidi C, Ehringer MA, Pitulle C, Pace NR. 2000. Phylogenetic-comparative analysis of the eukaryal ribonuclease P RNA. *RNA 6*:1895–1904.

Frank DN, Pace NR. 1998. Ribonuclease P: Unity and diversity in a tRNA processing ribozyme. *Annu Rev Biochem 67*:153–180.

Goldstein AL, McCusker JH. 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast 15*:1541–1553.

Guerrier-Takada C, Altman S. 1984. Catalytic activity of an RNA molecule prepared by transcription in vitro. *Science 223*:285–286.

Guerrier-Takada C, Altman S. 1986. M1 RNA with large terminal deletions retains its catalytic activity. *Cell 45*:177–183.

Hollingsworth MJ, Martin NC. 1987. Alteration of a mitochondrial tRNA precursor 5′ leader abolishes its cleavage by yeast mitochondrial RNase P. *Nucleic Acids Res 15*:8845–8860.

Houser-Scott F, Xiao S, Millikin CE, Zengel JM, Lindahl L, Engelke DR. 2002. Interactions among the protein and RNA subunits of *Saccharomyces cerevisiae* nuclear RNase P. *Proc Natl Acad Sci USA 99*:2684–2689.

Houser-Scott F, Ziehler WA, Engelke DR. 2001. *Saccharomyces cerevisiae* nuclear ribonuclease P: Structure and function. *Methods Enzymol 342*:101–117.

Jacobson MR, Cao LG, Taneja K, Singer RH, Wang YL, Pederson T. 1997. Nuclear domains of the RNA subunit of RNase P. *J Cell Sci 110*:829–837.

Jarrous N. 2002. Human ribonuclease P: Subunits, function, and intranuclear localization. *RNA 8*:1–7.

Jarrous N, Altman S. 2001. Human ribonuclease P. *Methods Enzymol 342*:93–100.

Jarrous N, Eder PS, Guerrier-Takada C, Hoog C, Altman S. 1998. Autoantigenic properties of some protein subunits of catalytically active complexes of human ribonuclease P. *RNA 4*:407–417.

Jarrous N, Reiner R, Wesolowski D, Mann H, Guerrier-Takada C, Altman S. 2001. Function and subnuclear distribution of Rpp21, a protein subunit of the human ribonucleoprotein ribonuclease P. *RNA 7*:1153–1164.

Jarrous N, Wolenski JS, Wesolowski D, Lee C, Altman S. 1999. Localization in the nucleolus and coiled bodies of protein subunits of the ribonucleoprotein ribonuclease P. *J Cell Biol 146*:559–572.

Jiang T, Altman S. 2001. Protein–protein interactions with subunits of human nuclear RNase P. *Proc Natl Acad Sci USA 98*:920–925.

Komine Y, Kitabatake M, Yokogawa T, Nishikawa K, Inokuchi H. 1994. A tRNA-like structure is present in 10Sa RNA, a small stable RNA from *Escherichia coli*. *Proc Natl Acad Sci USA 91*:9223–9227.

Lee JY, Engelke DR. 1989. Partial characterization of an RNA component that copurifies with *Saccharomyces cerevisiae* RNase P. *Mol Cell Biol 9*:2536–2543.

Lee JY, Evans CF, Engelke DR. 1991a. Expression of RNase P RNA in *Saccharomyces cerevisiae* is controlled by an unusual RNA polymerase III promoter. *Proc Natl Acad Sci USA 88*:6986–6990.

Lee JY, Rohlman CE, Molony LA, Engelke DR. 1991b. Characterization of RPR1, an essential gene encoding the RNA component of *Saccharomyces cerevisiae* nuclear RNase P. *Mol Cell Biol 11*:721–730.

Lindahl L, Fretz S, Epps N, Zengel JM. 2000. Functional equivalence of hairpins in the RNA subunits of RNase MRP and RNase P in *Saccharomyces cerevisiae*. *RNA 6*:653–658.

Ling MM, Robinson BH. 1997. Approaches to DNA mutagenesis: An overview. *Anal Biochem 254*:157–178.

Longtine MS, McKenzie A 3rd, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast 14*:953–961.

Lygerou Z, Mitchell P, Petfalski E, Seraphin B, Tollervey D. 1994. The POP1 gene encodes a protein component common to the RNase MRP and RNase P ribonucleoproteins. *Genes & Dev 8*:1423–1433.

Lygerou Z, Pluk H, van Venrooij WJ, Seraphin B. 1996. hPop1: An autoantigenic protein subunit shared by the human RNase P and RNase MRP ribonucleoproteins. *EMBO J 15*:5936–5948.

Mathews DH, Sabina J, Zuker M, Turner DH. 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol 288*:911–940.

Pagan-Ramos E, Lee Y, Engelke DR. 1996a. Mutational analysis of *Saccharomyces cerevisiae* nuclear RNase P: Randomization of universally conserved positions in the RNA subunit. *RNA 2*:441–451.

Pagan-Ramos E, Lee Y, Engelke DR. 1996b. A conserved RNA motif involved in divalent cation utilization by nuclear RNase P. *RNA 2*:1100–1109.

Pannucci JA, Haas ES, Hall TA, Harris JK, Brown JW. 1999. RNase P RNAs from some Archaea are catalytically active. *Proc Natl Acad Sci USA 96*:7803–7808.

Peck-Miller KA, Altman S. 1991. Kinetics of the processing of the precursor to 4.5 S RNA, a naturally occurring substrate for RNase P from *Escherichia coli*. *J Mol Biol 221*:1–5.

Pluk H, van Eenennaam H, Rutjes SA, Pruijn GJ, van Venrooij WJ. 1999. RNA–protein interactions in the human RNase MRP ribonucleoprotein complex. *RNA 5*:512–524.

Schmitt ME, Clayton DA. 1994. Characterization of a unique protein component of yeast RNase MRP: An RNA-binding protein with a zinc-cluster domain. *Genes & Dev 8*:2617–2628.

Srisawat C, Engelke DR. 2001. Streptavidin aptamers: Affinity tags for the study of RNAs and ribonucleoproteins. *RNA 7*:632–641.

Srisawat C, Engelke DR. 2002. RNA affinity tags for purification of RNAs and ribonucleoprotein complexes. *Methods 26*:156–161.

Srisawat C, Goldstein IJ, Engelke DR. 2001. Sephadex-binding RNA ligands: Rapid affinity purification of RNA from complex RNA mixtures. *Nucleic Acids Res 29*:E4.

Stolc V, Altman S. 1997. Rpp1, an essential protein subunit of nuclear RNase P required for processing of precursor tRNA and 35S precursor rRNA in *Saccharomyces cerevisiae*. *Genes & Dev 11*:2926–2937.

Stolc V, Katz A, Altman S. 1998. Rpp2, an essential protein subunit of nuclear RNase P, is required for processing of precursor tRNAs and 35S precursor rRNA in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA 95*:6716–6721.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improv-

ing the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res 22*:4673–4680.

Tranguch AJ, Engelke DR. 1993. Comparative structural analysis of nuclear RNase P RNAs from yeast. *J Biol Chem 268*:14045–14055.

Tranguch AJ, Kindelberger DW, Rohlman CE, Lee JY, Engelke DR. 1994. Structure-sensitive RNA footprinting of yeast nuclear ribonuclease P. *Biochemistry 33*:1778–1787.

van Eenennaam H, Jarrous N, van Venrooij WJ, Pruijn GJ. 2000. Architecture and function of the human endonucleases RNase P and RNase MRP. *IUBMB Life 49*:265–272.

van Eenennaam H, Lugtenberg D, Vogelzangs JH, van Venrooij WJ, Pruijn GJ. 2001a. hPop5, a protein subunit of the human RNase MRP and RNase P endoribonucleases. *J Biol Chem 276*:31635–31641.

van Eenennaam H, Pruijn GJ, van Venrooij WJ. 1999. hPop4: A new protein subunit of the human RNase MRP and RNase P ribonucleoprotein complexes. *Nucleic Acids Res 27*:2465–2472.

van Eenennaam H, van der Heijden A, Janssen RJ, van Venrooij WJ, Pruijn GJ. 2001b. Basic domains target protein subunits of the RNase MRP complex to the nucleolus independently of complex association. *Mol Biol Cell 12*:3680–3689.

Xiao S, Scott F, Fierke CA, Engelke DR. 2002. Eukaryotic ribonuclease P: A plurality of ribonucleoprotein enzymes. *Annu Rev Biochem 71*:165–189.

Ziehler WA, Morris J, Scott FH, Millikin C, Engelke DR. 2001. An essential protein-binding domain of nuclear RNase P RNA. *RNA 7*:565–575.

Zuker M, Mathews DH, Turner DH. 1999. Algorithms and thermodynamics for RNA secondary structure prediction: A practical guide. In: Barciszewski J, Clark BFC, eds. *RNA biochemistry and biotechnology.* Dordrecht, The Netherlands: Kluwer Academic Publishers. pp 11–43.