Role of the yeast Rrp1 protein in the dynamics of pre-ribosome maturation

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ABSTRACT

The *Saccharomyces cerevisiae* gene *RRP1* encodes an essential, evolutionarily conserved protein necessary for biogenesis of 60S ribosomal subunits. Processing of 27S pre-ribosomal RNA to mature 25S rRNA is blocked and 60S subunits are deficient in the temperature-sensitive *rrp1-1* mutant. We have used recent advances in proteomic analysis to examine in more detail the function of Rrp1p in ribosome biogenesis. We show that Rrp1p is a nucleolar protein associated with several distinct 66S pre-ribosomal particles. These pre-ribosomes contain ribosomal proteins plus at least 28 nonribosomal proteins necessary for production of 60S ribosomal subunits. Inactivation of Rrp1p inhibits processing of $27SA_3$ to $27SB_S$ pre-rRNA and of 27SB pre-rRNA to 7S plus 25.5S pre-rRNA. Thus, in the *rrp1-1* mutant, 66S pre-ribosomal particles accumulate that contain $27SA_3$ and $27SB_L$ pre-ribosomal RNAs.

Keywords: pre-ribosomes; pre-rRNA processing

INTRODUCTION

Eukaryotic ribosome biogenesis is an evolutionarily conserved process that begins in the nucleolus with transcription of rRNA precursors and that ends in the cytoplasm with the formation of the mature 40S and 60S ribosomal subunits (Woolford and Warner 1991; Eichler and Craig 1994; Venema and Tollervey 1999; Olson 2000; Raué 2003; Tsochner and Hurt 2003). During formation of mature ribosomal subunits, rRNA processing intermediates (pre-rRNAs) form a series of stable interactions with ribosomal proteins and transient interactions with nonribosomal proteins necessary for ribosome biogenesis. These interactions mediate modification of pre-rRNA, assembly of ribosomal proteins with the rRNA, structural rearrangements and nucleolytic processing of pre-rRNA, release of pre-ribosomal particles from the nucleolus to the nucleoplasm, export to the cytoplasm, and late cytoplasmic steps in maturation of functional ribosomal subunits (Raué 2003; Tsochner and Hurt 2003).

A series of pioneering experiments established that nascent pre-rRNA assembles with nonribosomal proteins and a subset of ribosomal proteins to form large ribosomal ribonucleoprotein particles (rRNPs), which are converted to mature ribosomal subunits (Warner and Soeiro 1967; Kumar and Warner 1972; Prestayko et al. 1974; Trapman et al. 1975; Trapman and Planta 1976; for review, see Hadjiolov 1985). These investigations established an ordered pathway of pre-ribosome formation. The 35S primary rRNA transcript is present in a 90S pre-ribosome. Following cleavage of the 35S pre-rRNA, 90S particles are converted into 66S and 43S assembly intermediates containing 27S and 20S pre-rRNAs, respectively. The 43S precursor particles are rapidly exported to the cytoplasm, where final steps of 40S subunit maturation occur, including production of mature 18S rRNA from 20S pre-rRNA (Udem and Warner 1973).

Subsequent analysis of eukaryotic pre-rRNA processing, particularly in the genetically tractable yeast *S. cerevisiae*, led to the discovery of a more complex pathway for processing of 27S pre-rRNA and the maturation of 66S pre-ribosomes (for review, see Kressler et al. 1999; Venema and Tollervey 1999). These studies revealed the existence of $27SA_2$, $27SA_3$, $27SB_S$, $27SB_L$, 25.5S, and 7S precursors to mature 25S and 5.8S rRNAs (Fig. 1). Thus it became evident that there must be at least four different 66S pre-ribosomes containing these different pre-rRNA precursors. 5S rRNA, transcribed separately from 35S pre-rRNA by RNA polymerase III, is thought first to associate with 66S pre-ribosomes containing 27SB pre-rRNA (Dechampesme et al. 1999).

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Article and publication are at http://www.rnajournal.org/cgi/doi/10.1261/rna.5255804.

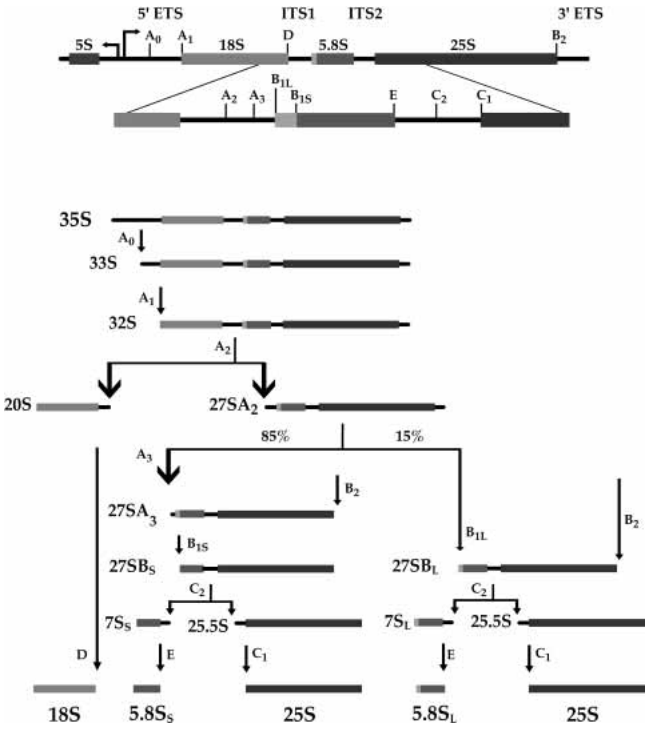

FIGURE 1. The pre-rRNA processing pathway in *Saccharomyces cerevisiae*. The 35S primary pre-rRNA transcript containing 18S, 5.8S, and 25S sequences flanked by transcribed spacers is produced by RNA polymerase I. The 5S pre-rRNA is separately transcribed by RNA polymerase III. Processing at the highlighted sites is mediated by several groups of endoribonucleases and exoribonucleases (not shown). Note that there are both major ($27SB_S$) and minor ($27SB_L$) pathways for production of 27SB pre-rRNA and subsequent processing to produce 25S plus 5.8S rRNA.

Recent technical advances in molecular biology and proteomics have enabled researchers to purify pre-ribosomal particles from yeast and to identify their protein as well as RNA constituents. These complexes include an active RNA polymerase I complex associated with rRNA processing and modification factors, 90S pre-ribosomes, a large U3 snoRNP complex, and several different 66S particles representing different stages of 60S subunit formation (Fath et al. 2000; Baßler et al. 2001; Harnpicharnchai et al. 2001; Saveanu et al. 2001; Dragon et al. 2002; Fatica et al. 2002; Grandi et al. 2002; Nissan et al. 2002; Schäfer et al. 2003). More than 140 nonribosomal proteins have been found in these pre-ribosomal complexes, including at least 60 proteins associated with the 66S precursor particles. In addition, a number of proteins not yet found in pre-ribosomal particles are implicated in biogenesis of 60S ribosomal subunits, based on their mutant phenotypes (Kressler et al. 1999; Venema and Tollervey 1999). Homologs of most of these yeast proteins were identified by proteomic analysis of proteins present in purified human nucleoli (Andersen et al. 2002; Scherl et al. 2002).

The *rrp1-1* mutation in yeast was the first mutation discovered that affects eukaryotic rRNA processing. The mutation was shown to cause an undefined defect in conversion of 27S pre-rRNA to mature 5.8S and 25S rRNAs, resulting in decreased levels of 60S ribosomal subunits (Andrew et al. 1976; Fabian and Hopper 1987; Gorenstein and Warner 1977). Consistent with this mutant phenotype, affinity purifications using several TAP-tagged nucleolar proteins led to the isolation of 66S pre-ribosomes containing 27S pre-rRNAs and a large set of nonribosomal proteins that included Rrp1p (Harnpicharnchai et al. 2001; Fatica et al. 2002; Gavin et al. 2002; Nissan et al. 2002; Saveanu et al. 2001). The human ortholog of Rrp1p is Nop52, a nucleolar protein that associates with pre-nucleolar bodies during nucleogenesis (Savino et al. 1999, 2001). Here we describe in more detail specific defects in 27S pre-rRNA processing caused by the *rrp1-1* mutation and relate these to the functional dynamics of pre-ribosome formation. The *rrp1-1* mutation blocks two steps in pre-rRNA processing: conversion of $27SA_3$ pre-rRNA to $27SB_S$ pre-rRNA, and cleavage of 27SB pre-rRNAs to generate 7S and 25.5S pre-rRNAs. In agreement with these pre-rRNA processing defects, we find that Rrp1p purifies with 66S pre-ribosomes containing $27SA_2$, $27SA_3$, 27SB, 25.5S, and 7S pre-rRNAs, as well as proteins necessary specifically for biogenesis of 60S ribosomal subunits.

RESULTS

Rrp1p is a conserved nucleolar protein

The evolutionary conservation of functions required for ribosome biogenesis is reflected in the high degree of amino acid sequence homology of many ribosome synthesis factors among eukaryotes (Andersen et al. 2002; Scherl et al. 2002). Homologs of Rrp1p are evident in *Schizosaccharomyces pombe, Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Mus musculus*, and *Homo sapiens* (Fig. 2; Savino et al. 1999). The amino-terminal 150 amino acids of Rrp1p homologs exhibit an average of 33% identity and 18% similarity. The metazoan homologs are considerably larger than the yeast proteins, extending an additional 100–335 amino acids at their carboxy termini.

The majority of proteins involved in ribosomal biogenesis, including Nop52, the human homolog of Rrp1p, are found primarily in the nucleolus, where rRNA is transcribed and most steps of rRNA processing and ribosome assembly occur (Kressler et al. 1999; Venema and Tollervey 1999; Olson et al. 2000). To establish the subcellular location of Rrp1p, we constructed a strain in which the wild-type *RRP1* gene was replaced by an *RRP1-GFP* fusion. This *RRP1-GFP* strain grew at rates identical to wild-type *RRP1* yeast and had no apparent deficits of 60S ribosomal subunits, indicating that the GFP-fusion protein is fully func-

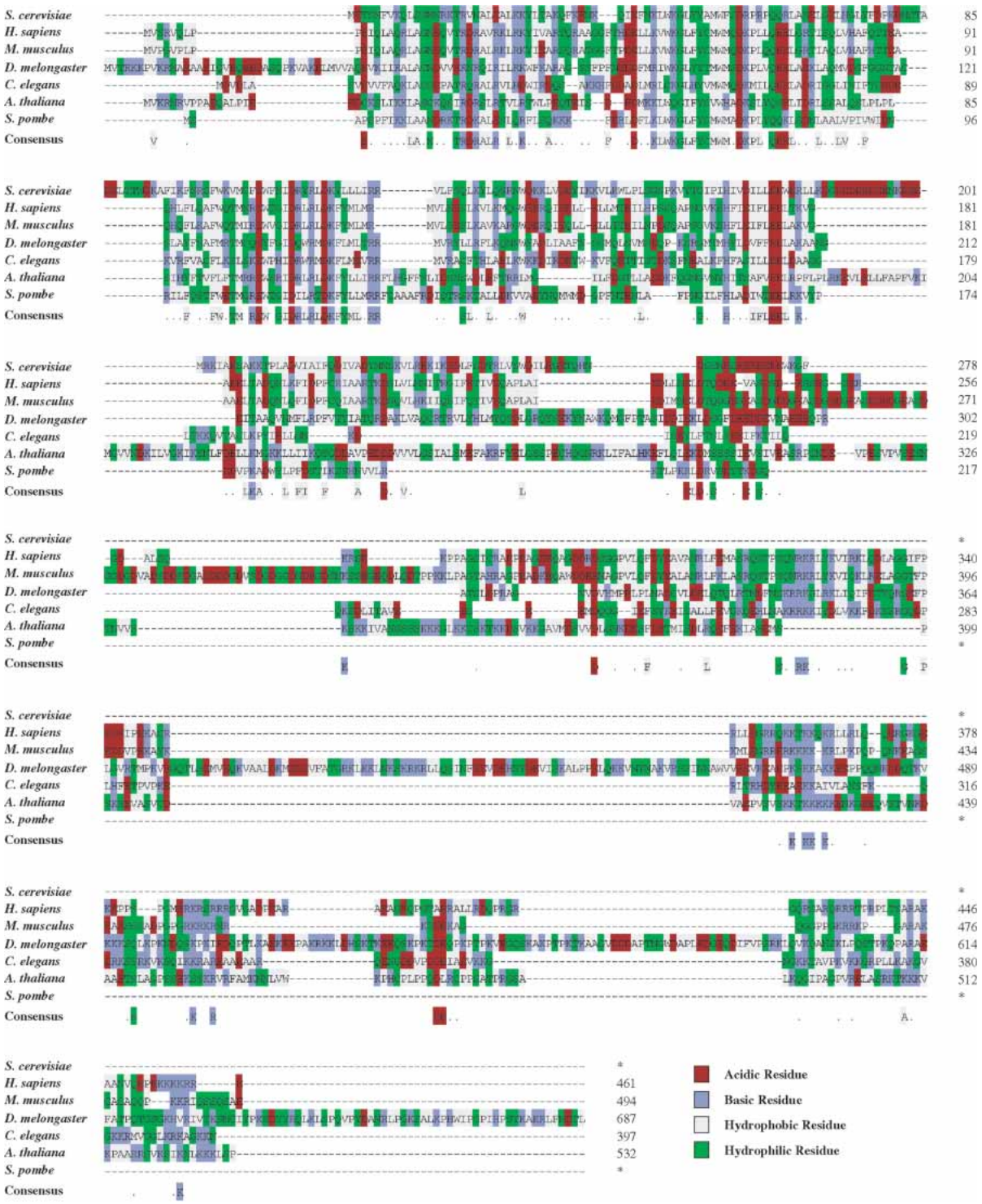

FIGURE 2. Amino acid sequence alignment of Rrp1p homologs. The program MacVector 6.5.3 (Oxford Molecular) was used to align *Saccharomyces cerevisiae* Rrp1p with *Homo sapiens* Nop52, *M. musculus* NNP-1, *Drosophila melanogaster* NNP-1, *Caenorhabditis elegans* C47E12.7, *Arabidopsis thaliana* AAL84969, and *Schizosaccharomyces pombe* SPBC9B6.07. Residues are colored according to their side-chain properties: acidic, red; basic, blue; hydrophobic, white; hydrophilic, green. A consensus sequence is indicated. Note that *S. cerevisiae* Rrp1p ends at F278 and therefore is considerably smaller than its metazoan homologs.

tional (data not shown). Examination by fluorescence microscopy of the cells expressing Rrp1p-GFP revealed a bright, crescent-shaped signal adjacent to the DAPI-stained nucleus and a fainter signal throughout the remainder of the cells (Fig. 3), indicating that Rrp1p is localized predominantly in the nucleolus.

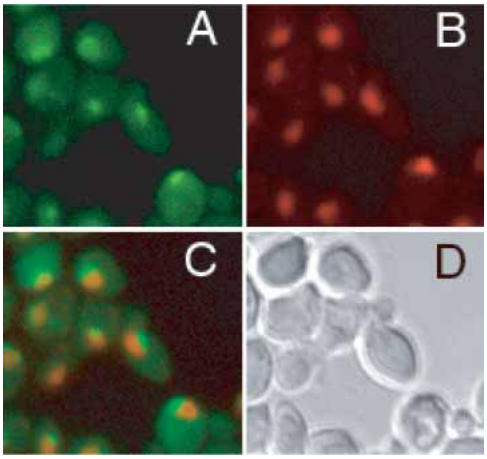

FIGURE 3. Rrp1p-GFP localizes to the nucleolus. Fluorescence microscopy was performed to detect Rrp1p-GFP in yeast strain JWY6155 (*RRP1-GFP*). (*A*) Fluorescence detected from Rrp1p-GFP. (*B*) DNA stained with 4′,6 diamidino-2-phenylindole (DAPI). (*C*) Superimposition of Rrp1p-GFP and DAPI signals. (*D*) Nomarski differential interference contrast image of the cells. (Pseudocolor was used to visualize Rrp1-GFP, green, and DAPI, red.)

Depletion or inactivation of Rrp1p leads to defects in biogenesis of 60S ribosomal subunits

Previously it was shown that the temperature-sensitive mutant *rrp1-1* is defective in the processing of 27S pre-rRNA to produce 25S rRNA and deficient in 32^P-labeled 60S ribosomal subunits (Andrew et al. 1976; Gorenstein and Warner 1977; Fabian and Hopper 1987). We sought to examine the function of Rrp1p in more detail by constructing and assaying a mutant strain containing a conditional null allele of *RRP1*, and by assaying pre-rRNA processing at higher resolution in *rrp1* mutants.

RRP1 is an essential gene (Giaever et al. 2002). A conditional null *rrp1* strain JWY6148 (*GAL-RRP1*) was constructed that contains a precise deletion of the chromosomal *RRP1* ORF and a low-copy *CEN* plasmid bearing *RRP1* under control of the *GAL* promoter. The *GAL-RRP1* construct was expressed in galactose-containing medium and repressed in glucose-containing medium. *RRP1* mRNA decreases to undetectable levels within 14 hours after the carbon-source shift of this *GAL-RRP1* strain (data not shown). The *GAL-RRP1* strain and the wild-type *RRP1* strain formed colonies of similar size on YEPGal solid medium (Fig. 4A, left), whereas the *GAL-RRP1* strain did not grow on YEP Glu medium (Fig. 4A, right). Twenty-two hours after shifting from YEPGal to YEPGlu liquid media, the growth rate of the *GAL-RRP1* strain slowed dramatically compared with that of the wild-type *RRP1* strain (Fig. 4B).

We assayed 40S and 60S ribosomal subunits, 80S ribosomes, and polyribosomes in the temperature-sensitive *rrp1-1* mutant and in the *GAL-RRP1* strain under permissive conditions (23°C or YEPGal) and after shifting to nonpermissive conditions (37° for 5 h or YEPGlu for 14 or 17 h). Levels of free 60S ribosomal subunits were diminished and amounts of free 40S ribosomal subunits increased in extracts from the shifted cells compared to unshifted cells or shifted wild-type cultures (Fig. 5; data not shown). "Halfmer" polyribosomes diagnostic of a shortage of 60S subunits (Helser et al. 1981; Rotenberg et al. 1988) were present in the mutants shifted to nonpermissive conditions (Fig. 5).

To analyze the steady-state levels of pre-rRNAs and mature rRNAs on depletion or inactivation of Rrp1p, we performed Northern blotting and primer extension analysis (Fig. 6). Amounts of mature 25S and 5.8S rRNAs were slightly decreased, relative to the U2 snRNA standard for loading, in the *GAL-RRP1* strain shifted to YEPGlu medium (Fig. 6B). The increased amounts of 27SB and 7S pre-rRNAs observed in the wild-type *RRP1* strain shifted to YEPGlu did not occur in the *GAL-RRP1* strain, but levels of $27SA_2$ plus $27SA_3$ pre-rRNAs remained high after the medium shift (Fig. 6B). 35S pre-rRNA also was increased rela-

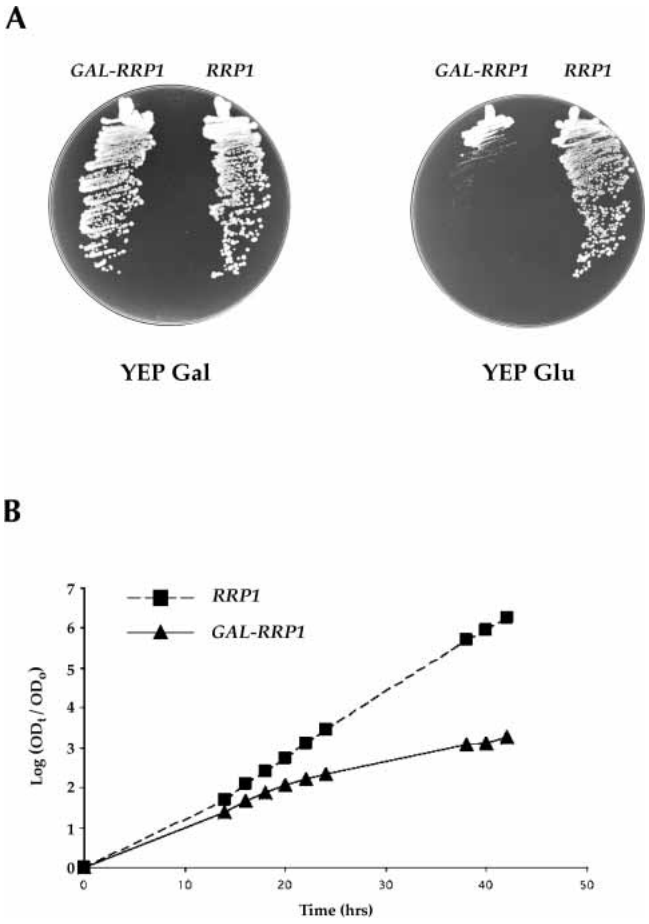

FIGURE 4. Growth of yeast cells is inhibited upon depletion of Rrp1p. (*A*) Strains JWY6148 (*GAL-RRP1*) and JWY6149 (*RRP1*) were streaked onto galactose- and glucose-containing solid YEP media and incubated at 30°C for 2 or 3 d, respectively. (*B*) Growth curve of JWY6148 (*GAL-RRP1*) and JWY6149 (*RRP1*) on shift from YEPGal liquid medium into YEPGlu liquid medium at 30°C; optical cell densities at 610 mm (Odt) were logarithmically plotted relative to the density of the starting culture (Odo) over time.

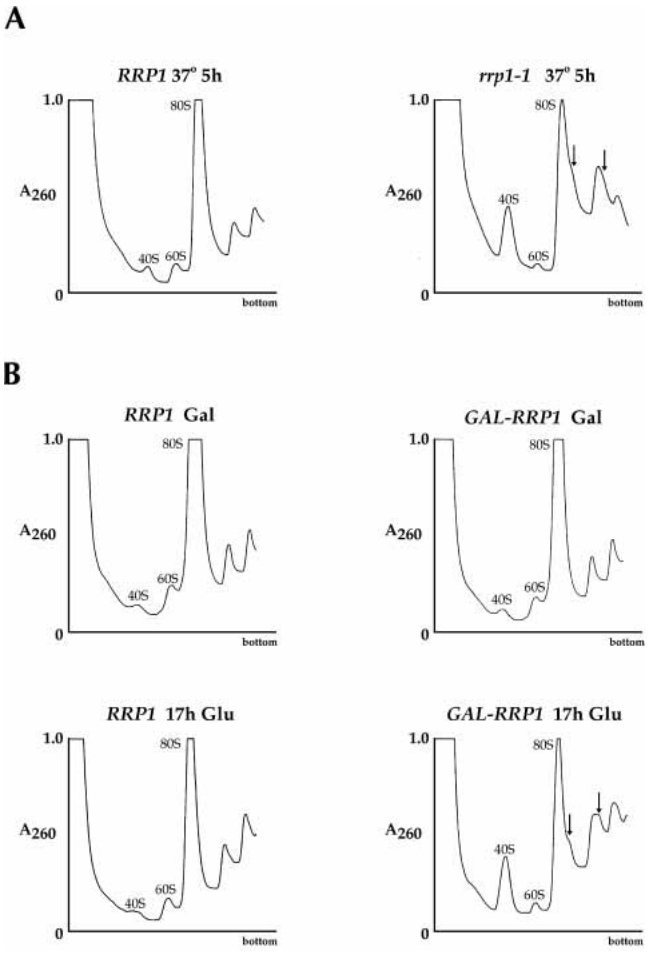

FIGURE 5. The *rrp1-1* mutation or depletion of Rrp1p causes a deficiency of free 60S ribosomal subunits. (*A*) Analysis of ribosomal subunits, ribosomes and polyribosomes in strains JWY6101 (*RRP1*, *left*) and JWY6102 (*rrp1-1*, *right*): Strains grown at 23°C were shifted to 37°C for 5 h. (*B*) Strains JWY6149 (*RRP1*, *left*) and JWY6148 (*GAL-RRP1*, *right*) were grown in YEPGal medium at 30°C (*top*) and shifted to YEPGlu medium at 30°C for 17 h (*bottom*). Whole-cell extracts were prepared, and ribosomes and polyribosomes were separated on 7%–47% sucrose velocity gradients. Peaks representing 40S and 60S ribosomal subunits and 80S monoribosomes are labeled; half-mer polyribosomes are indicated by arrows.

tive to 32S pre-rRNA, and consequently there was a decrease in amounts of 20S pre-rRNA and 18S rRNA. Primer extension assays more clearly revealed an increase in amounts of $27SA_3$ and, to a lesser extent, $27SA_2$ pre-rRNA, relative to 27SBs and $7S_S$ pre-rRNAs, on depletion of Rrp1p (Fig. 6C). However, amounts of $27SB_L$ plus $7S_L$ pre-rRNAs were only moderately decreased.

Pre-rRNA processing was similarly affected in the *rrp1-1* mutant shifted to 37°C for 5 h. Northern blotting and quantitation relative to the loading controls indicated that amounts of 25S, 5.8S, and 5S rRNA were approximately fourfold lower than in wild-type *RRP1* cells shifted to 37°C, and levels of 18S rRNA were approximately twofold lower. The most obvious defect was a drastic decrease in amounts of 7S and 25.5S pre-rRNAs. 35S and 20S pre-rRNAs accumulated, as well as the aberrant 23S intermediate, which is formed by premature cleavage at site A3 in 35S rRNA when processing at the A0, A1, and A2 sites is delayed. Primer extension indicated a strong accumulation of 27_S A3 pre-rRNA and an increase in $27SA_2$ relative to total B_L and B_S ends ($27SB_L$, $7S_L$, $27SB_S$, and $7S_S$ pre-rRNAs). In addition, amounts of $27SB_S$ plus $7S_S$ pre-rRNAs were dramatically decreased.

Kinetics of pre-rRNA processing in the *rrp1-1* mutant were analyzed by pulse-chase labeling in vivo and revealed effects similar to those observed by assays of steady-state levels of rRNAs. In the wild-type *RRP1* strain grown at 23°C or shifted to 37°C for 5 h, and in the *rrp1-1* mutant grown at 23°C, 27SA and 27SB pre-rRNAs were rapidly processed to mature 25S rRNA, and 20S pre-rRNA was quickly converted to 18S rRNA (Fig. 6D; data not shown). In contrast, in the *rrp1-1* mutant shifted from 23°C to 37°C for 5 h, $27SA_2$ and $27SA_3$ pre-rRNAs were produced to a lesser extent and with a slight delay, and little if any 27SB pre-rRNAs or mature 25S rRNA were evident (Fig. 6D). At early time points, levels of 35S pre-rRNA were higher in the shifted mutant strain than in control experiments. Formation of 18S rRNA was delayed and decreased in the mutant; significant amounts of 18S rRNA were not present until the 60-min chase point. 23S rRNA was evident in both the wild-type and mutant strains shifted to 37°C.

These measurements of steady-state levels of rRNA and assays of kinetics of pre-rRNA processing are consistent with observed deficiencies of production of 60S ribosomal subunits on depletion or inactivation of Rrp1p. Loss of functional Rrp1p primarily affects the major pathway of production of 27SB pre-rRNA by preventing conversion of $27SA_3$ pre-rRNA to $27SB_S$ pre-rRNA. In addition, in the absence of Rrp1p function, there is no compensatory increase in production of $27SB_L$ pre-rRNA through the minor pathway. Even though some $27SB_L$ pre-rRNA remained in the *rrp1-1* mutant after shifting to 37°C, no 7S or 25.5S pre-rRNA was detected. Thus, Rrp1p is required for two steps in pre-rRNA processing: conversion of the $27SA_3$ pre-rRNA to $27SB_S$ pre-rRNA and processing at the C2 site to convert 27SB pre-rRNA to 7S plus 25.5S pre-rRNAs.

Purification of 66S pre-ribosomes containing Rrp1p and identification of pre-rRNAs and proteins present in these particles

The nucleolar localization of Rrp1p, defects in processing of 27S pre-rRNAs exhibited by the *rrp1* mutants, and copurification of Rrp1p with other molecules in 66S pre-ribosomes (Harnpicharnchai et al. 2001; Fatica et al. 2002; Gavin et al. 2002; Nissan et al. 2002; Saveanu et al. 2001) indicate that Rrp1p associates with intermediates in the assembly of 60S ribosomal subunits. We investigated this in more detail by first examining whether Rrp1p cosediments

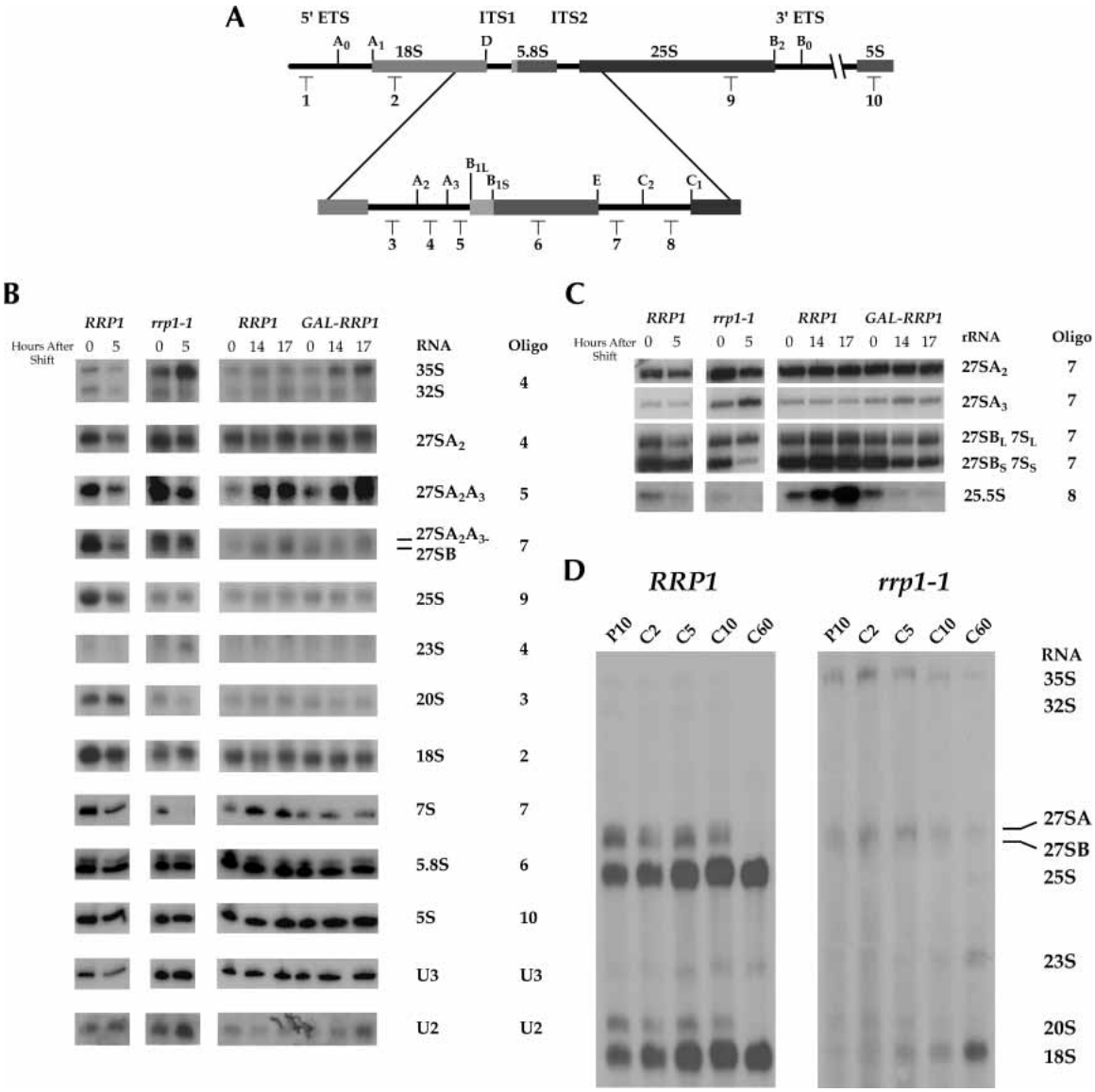

FIGURE 6. Processing of pre-rRNAs is perturbed in the *rrp1-1* mutant or on depletion of Rrp1p. (*A*) Sequences within the pre-rRNAs and the mature rRNAs complementary to the oligonucleotide probes are indicated. (*B*,*C*) Depletion of Rrp1p or the *rrp1-1* mutation affects the steady-state levels of mature rRNA and pre-rRNA. Strains JWY3400 (*RRP1*, *left*) and JWY6102 (*rrp1-1*, *right*) were grown at 23°C or shifted to 37°C for 5 h (*left*). Strains JWY6149 (*RRP1*, *left*) and JWY6148 (*GAL-RRP1*, *right*) were grown in YEPGal at 30°C and shifted to YEPGlu at 30°C for up to 17 h. RNA was extracted, resolved on agarose or acrylamide gels, and detected by (*B*) Northern hybridization or (*C*) primer extension. U2 snRNA and U3 snoRNA were used as loading controls. (*D*) The *rrp1-1* mutation disrupts kinetics of pre-rRNA processing and blocks accumulation of 25S rRNA. Strains JWY6101 (*RRP1*) and JWY6102 (*rrp1-1*) were grown at 23°C, shifted to 37°C for 5 h, and pulse-labeled with[5,6^{-3}H]-uracil for 10 min at 37°C. After the pulse, excess cold uracil was added and equal volumes of cells were collected for RNA extraction at different times of the chase. RNAs were resolved by gel electrophoresis and detected by autoradiography.

on sucrose gradients with 66S pre-rRNPs containing the 27S pre-rRNAs. To do so, we tagged Rrp1p at its carboxy terminus with the tandem affinity (TAP) tag containing the calmodulin binding peptide, a tobacco etch virus (TEV) protease cleavage site, and two protein A sequences (Rigaut et al. 1999). The *RRP1-TAP* strain JWY6144 grew at rates indistinguishable from wild-type yeast and contained wild-type levels of ribosomes, indicating that the tag did not visibly compromise the function of Rrp1p (data not shown). Western immunoblot analysis of sucrose gradient fractions from whole cell extracts demonstrated that Rrp1p-TAP sediments throughout most of the gradient, with a broad peak in fractions containing 60S ribosomal subunits and 80S ribosomes (Fig. 7). This sedimentation resembles that of several other proteins present in 66S pre-ribosomes containing Rrp1p, including Nop7p, Ssf1p, and Nsa3p (Harnpicharnchai et al. 2001; Fatica et al. 2002; Nissan et al. 2002) and corresponds to the position at which 66S pre-

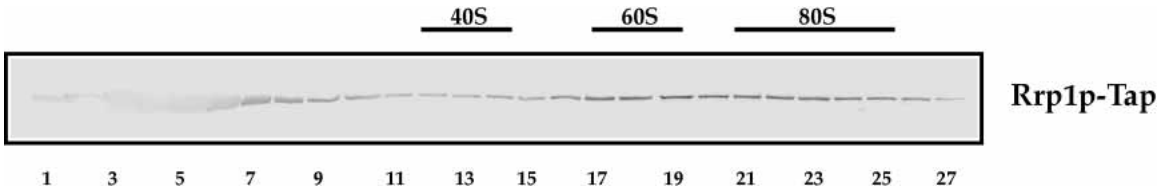

FIGURE 7. AP-tagged Rrp1p cosediments with 66S pre-ribosomes on sucrose velocity gradients. Whole-cell extracts were prepared from JWY6144 (*RRP1-TAP*) and fractionated on 7%–47% sucrose velocity gradients. Peaks representing 40S and 60S ribosomal subunits and 80S ribosomes are labeled. Fractions from the sucrose gradients were collected, and proteins were TCA-precipitated and subjected to immunoblot analysis to detect Tap-tagged Rrp1p.

ribosomes containing 27S or 25.5S and 7S pre-rRNAs fractionate (Trapman et al. 1975; Harnpicharnchai et al. 2001). Rrp1-TAP sedimenting in fractions lighter than the 66S particles might be present in smaller assembly subcomplexes, such as observed for other nucleolar proteins (P. Harnpicharnchai, E. Horsey, J. Jakovljevic, T. Miles, L. Tang, and J. Woolford, in prep.). Protein sedimenting in heavier fractions could be in aggregates, less soluble cell subfractions, or larger, uncharacterized complexes.

To further characterize the pre-ribosomes with which Rrp1p is associated, we identified the pre-rRNA and protein molecules that copurify under native conditions with TAP-tagged Rrp1p. Significant enrichment was observed for 27SB, 25.5S, and 7S pre-rRNAs, and less for 5.8S and 5S rRNAs, in the affinity-purified samples (Fig. 8). Although Northern analysis was unable to detect copurification of $27SA_2$ and $27SA_3$ pre-rRNAs, more sensitive primer extension assays did. There was no enrichment of 35S or 20S pre-rRNAs or mature 18S and 25S rRNAs. Very little if any of these RNAs were detected in mock purifications from the nontagged wild-type strain JWY3400, indicating the specificity of tandem affinity purification (Fig. 8, lane 3). Thus, Rrp1p is predominantly associated with 66S pre-ribosomes containing 27SB or 25.5S plus 7S pre-rRNAs, although Rrp1p is also present in the less abundant earlier particles containing $27SA_2$ or $27SA_3$ pre-rRNA.

Proteins that copurified with Rrp1p were identified using SDS–polyacylamide gel (PAGE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Sixty-one different proteins were found (Fig. 9), including 28 nonribosomal proteins previously implicated specifically in production of 60S ribosomal subunits (Table 1). Almost all of these proteins are essential, conserved among eukaryotes, localized predominantly in the nucleolus, and involved in one or more steps of processing of 27S pre-rRNAs (Table 1). In addition, the protein encoded by ORF YKL082c was found. This protein was previously discovered in 66S pre-rRNPs containing Ssf1p (Fatica et al. 2002) and is homologous to the mouse Surf-6 gene product, which is localized to the nucleolus (Magoulas and Fried 1996). Thus, Ykl082cp also is likely to be involved in ribosome biogenesis.

As expected for 66S precursors to 60S ribosomal subunits, 29 ribosomal proteins from the 60S ribosomal sub-

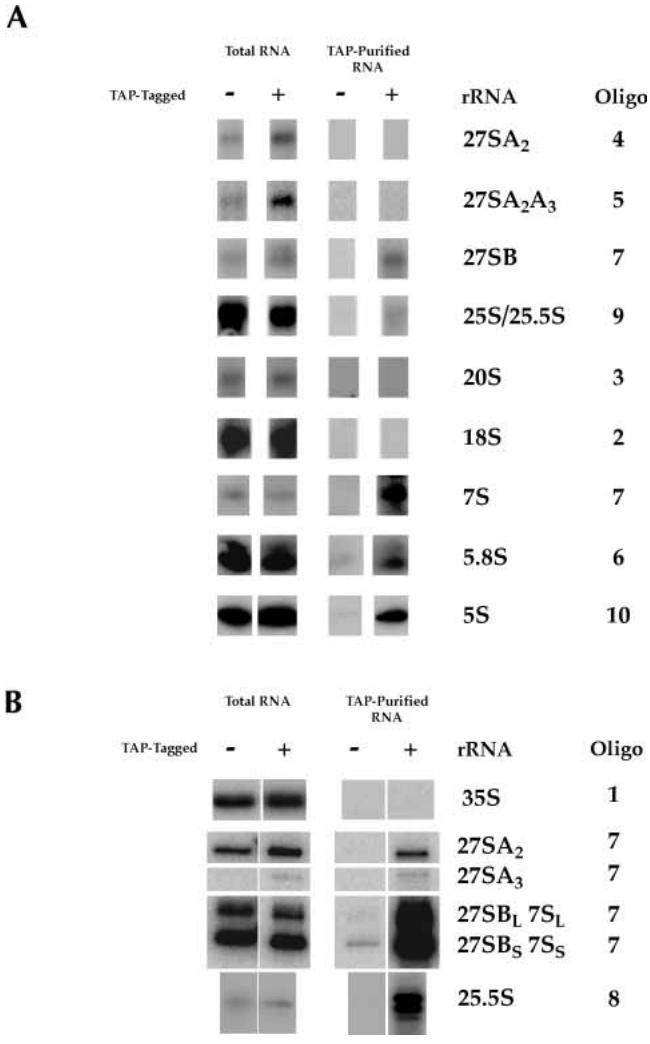

FIGURE 8. Rrp1p associates with pre-rRNAs in vivo. Whole-cell extracts were prepared from the *RRP1-TAP* strain JWY6144, and the untagged *RRP1* control strain JWY3400. RNA was extracted from whole cells and from the affinity-purified samples from both tagged and untagged strains. (*A*) RNAs were detected using Northern blotting with specific oligonucleotide probes complementary to pre-rRNA and mature rRNAs. (*B*) Primer extension was used to detect A_2, A_3, and B_L and B_S 5′ ends (27S plus 7S) as well as 25.5S and 35S pre-rRNAs. One hundred percent of TAP affinity-purified and 5% of total RNA were assayed.

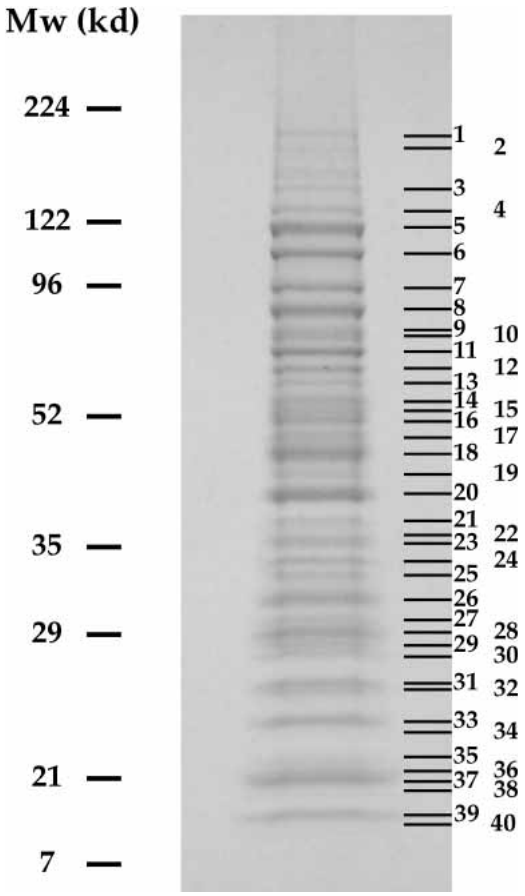

FIGURE 9. Rrp1p associates with ribosomal proteins and nonribosomal proteins necessary for production of 60S ribosomal subunits. Whole cell extracts were prepared from the *RRP1-TAP* strain JWY6144 and subjected to tandem affinity purification. Affinity-purified Rrp1p-TAP fractions were precipitated, resolved on a 4%–20% SDS-PAGE gel, and stained with Coomassie blue. Polypeptide bands were excised and identified by mass spectrometry (Tables 1, 2).

unit copurified with Rrp1p-TAP (Table 2). Similar results were obtained on purification of 66S pre-ribosomes using other TAP-tagged nucleolar proteins (Baßler et al. 2001; Harnpicharnchai et al. 2001; Saveanu et al. 2001; Fatica et al. 2002; Nissan et al. 2002; T. Miles and J. Woolford, in prep.). Three ribosomal proteins from the mature 40S ribosomal subunit were found (Table 2). Copurification of the 40S subunit ribosomal proteins may reflect the presence of small amounts of Rrp1p in the 90S pre-rRNP, which contains ribosomal proteins destined for both the small and the large ribosomal subunits (Grandi et al. 2002). Alternatively, this may reflect nonspecific association of these abundant ribosomal proteins with pre-ribosomes during the purification process (see Gavin et al. 2002). These data clearly demonstrate that Rrp1p stably associates with a core set of proteins present in 66S pre-ribosomal particles, each of which is necessary for biogenesis of 60S ribosomal subunits.

DISCUSSION

Rrp1p is an evolutionarily conserved nucleolar protein

Homologs of yeast Rrp1p exist across eukarya (this work and Savino et al. 1999). The human homolog Nop52 has been used as a marker to investigate reassembly of nucleoli after mitosis. Nop52 is recruited from the periphery of chromosomes to pre-nucleolar bodies and eventually to the nascent nucleolus. Nop52 and B23 are recruited after fibrillarin and nucleolin. It is believed that this order reflects the temporal function of each of these proteins in pre-rRNA processing (Savino et al. 1999, 2001). This is consistent with the nucleolar localization of yeast Rrp1p (Fig. 2) and with the requirement of Rrp1p for processing 27S pre-rRNAs (Fig. 6).

Rrp1p is important for two steps in pre-rRNA processing

The *rrp1-1* mutation was the first mutant allele found to specifically affect processing of eukaroytic pre-rRNA. Although it was shown that processing of 27S pre-rRNA to mature 25S and 5.8S rRNAs was blocked in the *rrp1-1* mutant, tools were not yet available to identify and distinguish the $27SA_2$, $27SA_3$, $27SB_S$, $27SB_L$, and 25.5S pre-rRNA processing intermediates (Andrew et al. 1976; Gorenstein and Warner 1977; Fabian and Hopper 1987). Thus, the exact nature of the 27S pre-rRNA processing defect in the *rrp1-1* mutant was not clear. Subsequent analysis has revealed a number of steps in the processing of 27S pre-rRNA to mature 25S and 5.8S rRNAs that occur in wild-type yeast (Venema and Tollervey 1999). $27SA_2$ pre-rRNA is produced on cleavage of the A_2 site in 32S pre-rRNA. $27SA_3$ and $27SB_L$ pre-rRNAs are generated from $27SA_2$ pre-rRNA by two mutually exclusive pathways (see Fig. 1). $27SA_2$ pre-rRNA is converted to $27SA_3$ pre-rRNA by endo-nucleolytic cleavage at the A_3 site mediated by the ribonucleoprotein RNase MRP (Schmitt and Clayton 1993). Two functionally interchangeable 5′ to 3′ exoribonucleases Rat1p and Xrn1p generate the 5′ end of $27SB_S$ pre-rRNA from $27SA_3$ pre-rRNA (Henry et al. 1994). The mechanism of production of $27SB_L$ pre-rRNA from $27SA_2$ pre-rRNA is unknown. Both $27SB_S$ and $27SB_L$ pre-rRNAs are cleaved at site C_2 by an unknown endoribonuclease to form the 7S and 25.5S pre-rRNAs. 7S pre-rRNA is processed into 5.8S rRNA by a complex of 11 different 3′ to 5′ exoribonucleases termed the exosome (Mitchell et al. 1997) and Rat1p and Xrn1p process 25.5S rRNA to 25S rRNA (Geerlings et al. 2000).

Here we used two different *rrp1* mutant alleles (*GAL-RRP1* and *rrp1-1*) and more specific probes for pre-rRNAs to investigate the role of Rrp1p in pre-rRNA processing. We observed that depletion (*GAL-RRP1*) or mutation (*rrp1-1*) of *RRP1* blocked conversion of $27SA_3$ pre-rRNA to $27SB_S$

TABLE 1. Nonribosomal proteins that copurify with Rrplp-TAP.

Gel identification number	Protein	ORF	Metazoan homolog	Essential	Sediments at 66S	Decreased 60S subunits	Location	Pre-RNA processing	Reference
20	Rrp1p	YDR087c	+	+	+	+	No	35S↑ 27SB↓ 27SA↑	Fabian and Hopper 1987; this work
2	Rrp5p	YMR229c	+	+	ND	+	No		Venema and Tollervey 1996
3	Mak21p/ Noc1p	YDR060w	+	+	+	+	No		Milkereit et al. 2001 Edskes et al. 1998
4	Dbp10p	YDL031w	+	+	ND	+	No		Burger et al. 2000
5	Erb1p	YMR049c	+	+	+	+	No	35↑27↓	Pestov et al. 2001
6	Drs1p	YLL008w	–	+	ND	+	No	27B↑	Ripmaster et al. 1992
7	Nop2p	YNL061w	+	+	+	+	No	35S↑27↑	de Beus et al. 1994
7	Nop4p	YPL043w	+	+	–	+	No	27S↓	Sun and Woolford 1994
7	Noc2p	YOR206w	+	+	+	+	No/N		Milkereit et al. 2001
8	Nop7p	YGR103w	+	+	+	+	No	$27 \rightarrow 25^{slow}$	Adams et al. 2002
9	Nog1p	YPL093w	+	+	ND	+	No	ND	J. Maddock, pers. comm.
11	Ebp2p	YKL172w	+	+	ND	+	No	$35S\uparrow 27SA_3\uparrow$	Huber et al. 2000; Tsujii et al. 2000
12	Ykl082p	YKL082c	+	+	ND	ND	ND	ND	Winzeler et al. 1999; this work
13	Ssf1p	YHR066w	+	+[a]	+	+	No	35S↑27SA↓	Kim and Hirsch 1998; Fatica et al. 2002
14	Has1p	YMR290c	+	+	ND	+/–	No	35S↑27SB↑	Emery et al. 2004
15	Ytm1p	YOR272w	+	+	+	+	No	ND	S. Matumoto and I. Yahara, pers. comm.; Harnpicharnchai et al. 2001; T.D. Miles and J.L. Woolford, Jr., in prep.
16	Nsa1p	YGL111w	–	+	ND	+	ND	ND	Winzeler et al. 1999; Harnpicharnchai et al. 2001
17	Mak16p	YAL025c	+	+	ND	+	ND	ND	Ohtake and Wickner 1995
18	Nsa3p	YHR052w	–	ND	ND	ND	ND	ND	Harnpicharnchai et al. 2001
19	Rlp7p	YNL002c	+	+	ND	+	No	$27SA_3\uparrow$	Dunbar et al. 2000
19	Rpf2p	YKR081c	+	+	+	+	No	27SB↑	Wehner and Baserga 2002; Morita et al. 2002
20	Rrp1p	YDR087c	+	+	+	+	No	35S↑, 27SB↓ $27SA_3\uparrow$	Fabian and Hopper 1987
22	Brx1p	YOL07c	+	+	ND	+	No	27S↓	Kaser et al. 2001
23	Rpf1p	YHR008W	+	+	ND	+	No	$27SA_3\uparrow$	Wehner and Berserga 2002
24	Nsa2p	YER126c	+	+	+	+	N	ND	Winzeler et al. 1999; Harnpicharnchai et al. 2001
24	Nop16p	YER002w	–	–	+	+	No/N	ND	Winzeler et al. 1999; Harnpicharnchai et al. 2001
25	Nop15p	YNL110c	–	+	+	+	No	ND	Winzeler et al. 1999; Harnpicharnchai et al. 2001
27	Loc1p	YFR001w	–	–	+	+	N	ND	Long et al. 2001; Harnpicharnchai et al. 2001
28	Tif6p	YPR016c	+	+	+	+	N/C	ND	Si and Maitra 1999; Basu et al. 2001; Ceci et al. 2004
33	Nip7p	YPL211w	+	+	+	+	No/N/C	ND	Zanchin et al. 1997

No, nucleolar; N, nuclear; C, cytoplasmic.
Protein bands 1, 10, 11, 14, and 21 were identified as Ura2p, Ssalp, Ssblp, Vma2p, Tdh3p. These proteins are common contaminants of affinity purifications were excluded from the table (Ho et al. 2002; Gavin et al. 2002).
[a]*SSF1* forms a redundant gene pair with *SSF2*. *ssf1*Δ/*ssf2*Δ double knock-outs are inviable, but single knockouts are not (Yu and Hirsch 1995).

TABLE 2. Ribosomal proteins that copurify with Rp1p-TAP.

Gel identification number	Protein(s)
18	Rpl3p
20	Rpl4p
23	Rpl5p
25	Rps3p
26	Rpl2p, Rpl8p, Rps1p
28	Rpl7p, Rpl15p
29	Rpll0p, Rpll3p, Rps8p
30	Rpl19p
31	Rpl6p, Rpl18p
32	Rpl6p, Rpl17p, Rpl20p
33	Rpll1p, Rpl21p, Rpl24p
34	Rpl12p
35	Rpl28p
36	Rpl25p
37	Rpl26p, Rpl27p, Rpl32p
38	Rpl23p
39	Rpl33p, Rpl36p
40	Rpl30p, Rpl43p

pre-rRNA and prevented cleavage of 27SB pre-rRNAs at the C_2 site to produce 25.5S plus 7S pre-rRNAs (Fig. 6). Because depletion or mutation of *RRP1* produce similar defects in pre-rRNA processing, the *rrp1-1* mutation may act as a null allele in vivo. This is consistent with our inability to detect Rrp1p in affinity purified pre-ribosomal complexes in the temperature-sensitive mutant strain (P. Harnpicharnchai, E. Horsey, J. Jakovljevic, T. Miles, L. Tang, and J. Woolford, in prep.). The *ebp2*, *rlp7*, and *rpf1* mutants have pre-rRNA processing defects strikingly similar to that of the *rrp1-1* mutant (Huber et al. 2000; Dunbar et al. 2000; Gadal et al. 2002; Wehner and Baserga 2002). Thus, Rrp1p, Ebp2p, Rlp7p, and Rpf1p form a subset of proteins that might mediate structural changes in 66S pre-ribosomes necessary for processing at site A_3 by Rat1p/Xrn1p, cleavage at the C_2 site, and subsequent processing by Rat1p/Xrn1p and the exosome. Each of these proteins is present in 66S pre-rRNPs containing Rrp1p (Table 1).

Rrp1p associates with 66S pre-ribosomal particles

Our results indicate that Rrp1p associates primarily with 66S pre-ribosomal particles containing 27SB, 25.5S, and 7S pre-rRNAs. The lesser amounts of $27SA_2$ and $27SA_3$ pre-rRNAs copurifying with Rrp1p are consistent with the fact that these pre-rRNAs are shorter-lived than 27SB pre-rRNAs and thus are present in a smaller fraction of total 66S pre-rRNPs than those containing 27SB, 25.5S, and 7S pre-rRNAs. The lower amount of 27SA pre-rRNAs in Rrp1p-containing particles relative to total cellular 27SA pre-rRNAs suggests that Rrp1p may not be present in all 66S rRNPs containing 27SA pre-rRNAs. The lack of association of Rrp1p with mature 25S rRNA but the presence of mature 5.8S rRNA most likely reflects independent processing events that are necessary to form these rRNAs after C_2 cleavage. 5.8S rRNA may be formed from 7S pre-rRNA faster than 25.5S pre-rRNA is converted into 25S rRNA. Therefore, our data indicate that Rrp1p first associates with 66S pre-ribosomes at some point after the formation of the $27SA_2$ pre-rRNA. Rrp1p is present in subsequent 66S pre-rRNPs containing $27SA_3$ pre-rRNA, then 27SB pre-rRNAs and 5S rRNA, and dissociates from 66S pre-ribosomes sometime after the cleavage at site C_2 and processing of 25.5S and 7S pre-rRNAs into mature rRNAs.

In addition to determining the RNAs present in 66S pre-ribosomes containing Rrp1p, we identified the protein components of these particles. The set of nonribosomal proteins associated with Rrp1p closely overlaps with the proteins that copurify with TAP-tagged Nop7p, Ssf1p, and Ytm1p (Harnpicharnchai et al. 2001; Fatica et al. 2002; Gavin et al. 2002; Ho et al. 2002; T. Miles and J. Woolford, in prep.). This set of approximately 30 nonribosomal proteins includes many bona fide 60S ribosomal subunit assembly factors that, on depletion or inactivation, cause specific defects in the processing of 27S pre-rRNA (see above; Table 1), consistent with the presence of these pre-rRNAs in the Rrp1p-containing particles. These assembly factors include a putative methylase Nop2p (Hong et al. 1997); RNA binding proteins Nop4p, Rpf1p, and Rpf2p (Sun and Woolford 1994; Wehner and Baserga 2002); putative RNA helicases Drs1p, Dbp10p, and Has1p (Ripmaster et al. 1992; Burger et al. 2000; Emery et al. 2004); putative scaffolding proteins Erb1p and Ytm1p (Pestov et al. 2001; T. Miles and J. Woolford, in prep.); the putative GTPase Nog1p (Saveanu et al. 2001); and a potential molecular switch Tif6p (Senger et al. 2001), as well as many proteins with no predicted specific molecular function.

Additional interesting information is also gleaned from the types of proteins not found associated with Rrp1p. Other than several ribosomal proteins destined for the 40S subunit, we did not find any proteins specifically required for biogenesis of 40S ribosomal subunits. Consistent with the set of pre-rRNAs associated with Rrp1p, we found no proteins involved in late nucleoplasmic stages of 66S pre-ribosome maturation and export, such as Rix1p, Sda1p, Arx1p, or Nmd3p (Gadal et al. 2001; Nissan et al. 2002), nor did we find cytoplasmic factors such as Kre35p (Nissan et al. 2002). Most remarkable is the absence of ribonucleases and cofactors that directly mediate the removal of the transcribed rRNA spacer elements. These missing factors include the exosome complex that processes 7S pre-rRNA to 5.8S rRNA (Mitchell et al. 1997), the exoribonucleases Rat1p and Xrn1p that produce the 5′ ends of 5.8S and 25S rRNAs (Henry et al. 1994), and RNase MRP that mediates formation of $27SA_3$ pre-rRNA (Schmitt and Clayton 1993). The absence of these bona fide RNA processing factors and the presence of many other nucleolar proteins copurifying with Rrp1p suggest that these complexes may represent a structural core of 66S pre-ribosomal particles. The RNA

processing factors might form transient relationships with these particles to remove the transcribed spacer RNA, and therefore do not copurify with 66S pre-ribosomes in detectable quantities. It will be of interest to seek mutant forms of processing factors that may stably associate with pre-ribosomes.

MATERIALS AND METHODS

Strains, plasmids, and media

Cells were grown at 30°C in YEPGlu (1% extract, 2% peptone, 2% glucose), YEPGal (1% yeast extract, 2% peptone, 2% galactose), or synthetic media (Sherman et al. 1986), unless otherwise noted. Yeast cells were transformed with DNA using the lithium acetate method (Ito et al. 1983).

The conditional null allele *GAL-RRP1* was constructed as follows: the *RRP1 ORF* was amplified by PCR from the plasmid YEP*RRP1* (Fabian and Hopper 1987), using primers EWH37 and EWH38 containing BamHI sites, digested with BamHI, and cloned into the BamHI site immediately 3′ of the *GAL1* promoter in plasmid pBM258T (Johnston and Davis 1984). The resulting plasmid pJW5269 (*GAL-RRP1*) also contains the *URA3* gene. The sequence and orientation of the *RRP1 ORF* were confirmed by sequencing.

To construct strain JWY6148 (*GAL-RRP1*) containing the genomic null allele *rrp1*Δ::*KanMX4* and the plasmid-borne conditional null allele *GAL-RRP1*, diploid strain JWY6145 (*RRP1/rrp1*Δ::*kanMX4*, Research Genetics Record 24022) was transformed with plasmid pJW5269 (*GAL-RRP1*). The resulting strain was sporulated, tetrads were dissected, and a G418 (geneticin) resistant Ura+ spore (JWY6148) was identified. JWY6149 (*RRP1*) was derived from the same tetrad as JWY6148, but it contains the chromosomal wild-type *RRP1* gene and does not contain the plasmid pJW5269.

The TAP-tag was placed at the 3′ end of the *RRP1* gene to construct strain JWY6144 (*RRP1-TAP*) as follows: the TAP-tag and the *Kluyveromyces lactis TRP1* gene were amplified by PCR from plasmid pBS1539 (Rigaut et al. 1999), using two primers (EWH33 and EWH34) containing sequences adjacent to the 3′ end of the *RRP1* ORF. The resulting PCR product was transformed into the *trp1-1* strain JWY3400. Integration of the TAP-tag cassette in-frame with the last codon of the *RRP1* ORF was confirmed via Western immunoblotting and genomic PCR.

To construct strain JWY6155 (*RRP1*-GFP) expressing a fusion of the green fluorescent protein GFP to the carboxy terminus of Rrp1p, two primers that include sequences complementary to the 3′ end of the *RRP1* ORF (RRP1up, RRP1down) were used to PCR amplify a GFP (S65T) fusion construct sequence from plasmid pFA6a-GFP(S65T)-*HIS*3MX6 (Longtine et al. 1998). The resulting PCR product (containing the *HIS3* ORF) was transformed into yeast strain JWY6147 that contains the *his3*Δ*200* allele. His+ transformants were screened by microscopy for expression of GFP. All strains used in this work are listed in Table 3.

Analysis of RNA

RNA was extracted from strains JWY3400 (*RRP1*) and JWY6102 (*rrp1-1*) grown in YEPGlu at permissive temperature to approximately 1×10^7 cells/mL, diluted, and shifted to 37°C for 5 h to approximately 1×10^7 cells/mL. Strains JWY6149 (*RRP1*) and JWY6148 (*GAL-RRP1*) were grown in YEPGal at 30°C to approximately 1×10^7 cells/mL, diluted, and shifted to YEPGlu at 30°C for up to 17 h to approximately 1×10^7 cells/mL. RNA was extracted with phenol, precipitated with ethanol, suspended in RNAse-free water, and mixed with two volumes of sample buffer (8% formaldehyde, 1.3× MOPS buffer, 65% formamide, 0.02% xylene cyanol, and 0.1% bromophenol blue). Samples were subjected to electrophoresis on a 23-cm 1.2% agarose gel containing 6% formaldehyde and 1X MOPS buffer [1 mM sodium EDTA, 20 mM 3–(N-morpholino propane sulfonic acid pH 7.5, 8 mM sodium acetate)] for 24 h at 55 V or 4.4–6 h at 150 V with continuous recirculation of 1X MOPS buffer. RNAs were visualized using ethidium bromide (0.5 μg/mL). Following electrophoresis, gels were washed in 1X TBE for 10 min, and RNA was transferred by capillary action from the gel to a GeneScreen Plus membrane (NEN). Blots were preincubated for at least 2 h at 65°C in hybridization buffer: bovine serine albumin (10 mg/mL), 0.5 M sodium phosphate buffer ($Na_2HPO_4 \cdot 7H_2O/H_3PO_4$ pH 7.2), 1 mM EDTA pH 8, and 5% SDS. Oligonucleotides were end-labeled using 32Pγ-ATP and polynucleotide kinase and hybridized to blotted RNAs at 47°C–48°C overnight in fresh hybridization buffer. Blots were washed three times at room temperature in 6X SSC (3 M NaCl, 300 mM Na citrate, pH 7.0) and 0.2% SDS and were exposed to X-ray film. Oligonucleotide probes or primers are listed in Table 4.

Small–molecular weight RNA was suspended in RNase-free water, mixed with an equal volume of sample buffer (0.1X TBE buffer, 10 M urea, 0.1% xylene cyanol, 0.1% bromophenol blue),

TABLE 3. Strains used in this study.

Strain	Genotype	Source
JWY6101	*MATa his7 ade2 lys2 trp1 tyr1 leu1 gal2 gal7 RRP1*	A. Hopper
JWY6102	*MATa his7 ade2 lys2 trp1 tyr1 gal1 gal2 rrp1-1*	A. Hopper
JWY 6145	*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 rrp1Δ::KanMX4/RRP1*	Research Genetics
JWY6148	*MATa his3Δ1 leu2Δ0 LYS2 MET15 ura3Δ0 rrplΔ::KanMX4* plus pJW5269 (*GALl-RRP1 URA3*)	This study
JWY6149	*MATa his3Δ1 leu2Δ0 lys2Δ0 MET15 ura3Δ0 RRP1*	This study
JWY3400	*MATa ura3-52 lys2-801 trp1-1 leu2-1 his3Δ200 pep4::HIS3 prb1Δ 1.6R can1 RRPl*	E. Jones
JWY6144	*MATa ura3-52 lys2-801 trp1-1 leu2-1 his3Δ200 pep4::HI S3 prb1Δ 1.6R can1 RRP-1 TAP TRPl1*	This study

TABLE 4. Oligonucleotides used in this study.

1	5′-ACACGCTGTATAGAGACTAGGC-3′
2	5′-CATGGCTTAATCTTTGAGAC-3′
3	5′-GCTCTCATGCTCTTGCC-3′
4	5′-TGTTACCTCTGGGCCC-3′
5	5′-AATTTCCAGTTACGAAAATTCTTGT-3′
6	5′-TTTCGCTGCGTTCTTCATC-3′
7	5′-GGCCAGCAATTTCAAGTTA-3′
8	5′-CGCCTAGACGCTCTCTTCTTA-3′
9	5′-CTCCGCTTATTGATATGC-3′
10	5′-GGTCACCCACTACACTACTCGG-3′
U2	5′-GGACATAAACGGCTCGG-3′
U3	5′-GGATTGCGGACCAAGCTAA-3′
EWH33	5′-TCCAGTAACGAATCTGAAGAAGAGGAAGAGGAAGAGTGGAAGGGATTTTCCATGGAAAAGAGAAG-3′
EWH34	5′-ATTTATATGAGCGACTTACGATTACTTACATACTACCGCCAGGAAGAATACGACTCACTATAGGG-3′
EWH37	5′-CGCGGATCCGCATTCACGGCATAATACAC-3′
EWH38	5′-GGCGGATCCGGACAATACACGCCAATCTG-3′
RRP1up	5′-TCCAGTAACGAATCTGAAGAAGAGGAAGAGGAAGAGTGGAAGGGATTTCGGATCCCCGGGTTAATTAA-3′
RRP1down	5′-ATTTATATGAGCGACTTACGATTACTTACATACTACCGCCAGGAAGAAGAATTCGAGCTCGTTTAAAC-3′

and subjected to electrophoresis on a 20-cm 1 × TBE, 5% acrylamide 7 M urea gel for 4 h at 15 milliamps. Immediately following electrophoresis, gels were washed in 0.5X TBE for 10 min and electroblotted to a Nytran N membrane (Schleicher & Schuell).

Pulse-chase analysis of rRNA was performed as described by Venema et al. (1998) with the following modifications: strains JWY6101 (*RRP1*) and JWY6102 (*rrp1-1*) grown in YEPGlu at 23°C to approximately 5×10^6 cells/mL were shifted to 37°C for 5 h, pulse-labeled with 18 uCi/mL of [5,6–3H]-uracil for 10 min, and chased with excess cold uracil (2 mM) for up to 60 min. RNA was extracted and equal counts per minute were subjected to electrophoresis on 1.2% agarose gels. RNA was capillary transferred to Gene Screen. Membranes were exposed to phosphorimaging screens (Amersham) for analysis using Molecular Dynamics' Image Quant.

Affinity purification and mass spectrometry

Yeast strain JWY6144 (*RRP1-TAP*) was grown in YEPGlu at 23°C to approximately 2×10^7 cells/mL, and cell-free extracts were prepared as described in Harnpicharnchai et al. (2001). Affinity purification of TAP-tagged Rrp1p and associated molecules was performed as described in Rigaut et al. (1999).

Coenriched RNAs were extracted from the final eluate with phenol/chloroform and isoamyl alcohol, precipitated with ethanol, and identified by Northern analysis or primer extension (Venema et al. 1998).

Proteins were TCA-precipitated from the final eluate, suspended in SDS sample buffer, and separated by SDS-PAGE on 4%–20% or 14% Novex gels. Proteins were stained with the Colloidal Blue Staining Kit (Invitrogen) and excised, reduced, alkylated, and subjected to tryptic digestion using the Investigator ProGest (Genomic Solutions), per manufacturer's instructions. Resulting peptides were desalted and suspended in 50% acetonitrile, 0.1% formic acid, using μ-C18 ZipTips (Millipore) per manufacturer's instructions. Desalted samples were mixed with a 50% acetonitrile, 0.05% TFA solution saturated with matrix (a-cyano-4-hydroxycinnamic acid). Peptide mass sets were determined using an Applied Biosystems Voyager DE-STR matrix-assisted laser desorption ionization time of flight mass spectrometer and analyzed with Data Explorer software (Applied Biosystems) using tryptic autolysis peaks and fibrinopeptide B (GLU1) for internal calibration. Mass values were used to search databases (PROWL http://prowl.rockefeller.edu and MASCOT http://www.matrixscience.com).

Sucrose gradient analysis

Strains JWY6101 (*RRP1*) and JWY6102 (*rrp1-1*) were grown at 23°C in YEPGlu and shifted to 37°C for 5 h to approximately 2×10^7 cells/mL. Strains JWY6149 (*RRP1*) and JWY6148 (*GAL-RRP1*) were grown in YEPGal at 30°C to approximately 2×10^7 cells/mL, diluted, and shifted to YEPGlu at 30°C up to 17 h to 2×10^7 cells/mL. Ribosomes, pre-ribosomes, and polyribosomes were fractionated on sucrose gradients (Deshmukh et al. 1993). One-milliliter fractions were collected and TCA-precipitated for Western immunoblot analysis.

Western immunoblotting

Immunoblotting was performed according to standard protocols (Ausubel et al. 1994). Alkaline phosphatase-conjugated to IgG (to detect TAP-tagged Rrp1p, Pierce) was used to probe OPTITRAN nitrocellulose membranes (Schleicher & Schuell).

Fluorescence microscopy

Strain JWY6155 (*RRP1-GFP*) was grown at 30°C to approximately 8×10^6 cells/mL, harvested, and prepared for microscopy (Pringle et al. 1989). Cells were stained with 4′,6 diamidino-2-phenylindole (DAPI) for 3.5 min to detect nuclear DNA. Slides were viewed using a fluorescence microscope (Carl Zeiss) equipped with a 100× objective. Photographs were taken on a Hamamatsu black-and-white charge-coupled-device-coupled camera. Digital images were acquired using Adobe Photoshop.

ACKNOWLEDGMENTS

We are grateful to Mark Longtine (Oklahoma State University) for plasmids used to disrupt or tag genes. We thank Susan Dowd (Carnegie Mellon University) and Mark Bier (Carengie Mellon University) for assistance with mass spectrometry and Anita Hopper (Penn State University) for the original *rrp1-1* mutant strain. We thank Elizabeth Jones, Lasse Lindahl, Javier Lopez, Jon Minden, Gordon Rule, and members of our laboratory for advice about experiments and for comments on the manuscript. This work was supported by National Institutes of Health grants R01 GM28301 to J.L.W., F31 GM19937 to E.W.H., and F31 GM65067 to T.D.M., and by funds from the government of Thailand provided to P.H.

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Received December 3, 2003; accepted February 2, 2004.

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