The 18S rRNA dimethylase Dim1p is required for pre-ribosomal RNA processing in yeast

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The $m_2^6A_{1779}m_2^6A_{1780}$ dimethylation at the 3′ end of the small subunit rRNA has been conserved in evolution from bacteria to eukaryotes. The yeast 18S rRNA dimethylase gene *DIM1* was cloned previously by complementation in *Escherichia coli* and shown to be essential for viability in yeast. A conditional *GAL10::dim1* strain was constructed to allow the depletion of Dim1p from the cell. During depletion, dimethylation of the pre-rRNA is progressively inhibited and pre-rRNA processing at cleavage sites A1 and A2 is concomitantly lost. In consequence, the mature 18S rRNA and its 20S precursor drastically underaccumulate. This has the effect of preventing the synthesis of nonmethylated rRNA. To test whether the processing defect is a consequence of the absence of the dimethylated nucleotides or of the Dim1p dimethylase itself, a *cis*-acting mutation was created in which both dimethylated adenosines are replaced by guanosine residues. Methylation cannot occur on this mutant pre-rRNA, but no clear pre-rRNA processing defect is seen. Moreover, methylation of the wild-type pre-rRNA predominantly occurs after cleavage at sites A1 and A2. This shows that formation of the $m_2^6A_{1779}m_2^6A_{1780}$ dimethylation is not required for pre-rRNA processing. We propose that the binding of Dim1p to the pre-ribosomal particle is monitored to ensure that only dimethylated pre-rRNA molecules are processed to 18S rRNA.

Key Words: Methylation; RNA processing; ribosome synthesis; rRNA; yeast]

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Ribosomes have a complex biosynthetic pathway requiring the processing and modification of the ribosomal RNAs (rRNAs) and their assembly with ribosomal proteins (r-proteins) (for review, see Warner 1989; Raué and Planta 1991; Woolford and Warner 1991; Woolford 1991). In eukaryotic cells, most steps in ribosome synthesis occur in a specialized nuclear compartment, the nucleolus.

The four mature rRNA species are produced both by RNA polymerase I and polymerase III (Pol I and Pol III). Pol I transcribes a large precursor (the 35S pre-rRNA in yeast) that undergoes a series of endo- and exonucleolytic processing reactions to yield three mature rRNA species, the 18S rRNA component of the 40S ribosomal subunit, and the 5.8S and 25S rRNA components of the 60S ribosomal subunit. The structure of the yeast rDNA genes and a current model of the major yeast pre-rRNA processing pathway are presented in Figure 1. The remaining rRNA species, the 5S rRNA component of the 60S ribosomal subunit, is transcribed by Pol III.

Shortly after transcription, the pre-RNA is modified both by methylation and formation of pseudouridine, with all known pre-rRNA modifications occurring in the mature rRNA regions. In yeast, most of the 67 methyl groups are linked to the 2′-*O* of ribose moieties but 12 are attached to bases (6 in each of the 18S and 25S rRNAs). Base modification, unlike 2′-*O*-methylation, occurs mainly at later stages in ribosome biogenesis (Brand et al. 1977; Klootwijk and Planta 1989). Almost all of the modified nucleotides that have been localized lie in the evolutionarily conserved regions of the rRNAs that are brought together in the functional center of the ribosome (for review, see Maden 1990; Brimacombe 1993).

Many of the methylated nucleotides present in eukaryotic rRNA are conserved from yeast to humans, arguing that they play some function that is both conserved and important. However, in no case is it known what role these modifications actually play in either the biogenesis or function of eukaryotic ribosomes. The requirement for methylation to allow the processing of eukaryotic pre-rRNA is also unclear. Methionine deprivation of HeLa cells leads to failure of the final stages of rRNA maturation (Vaughan et al. 1967). However, inhibition of methylation by treatment with ethionine, a competitive inhibitor of *S*-adenosylmethionine biosynthesis, allows partial (Swann et al. 1975; Wolf and Schlessinger 1977) or complete (Caboche and Bachellerie 1977) processing at the normal sites although with low efficiency. In yeast, the *nop1-3* mutant, which is inhibited in nucleolar pre-rRNA methylation, does not block pre-rRNA processing (Tollervey et al. 1993). Moreover,

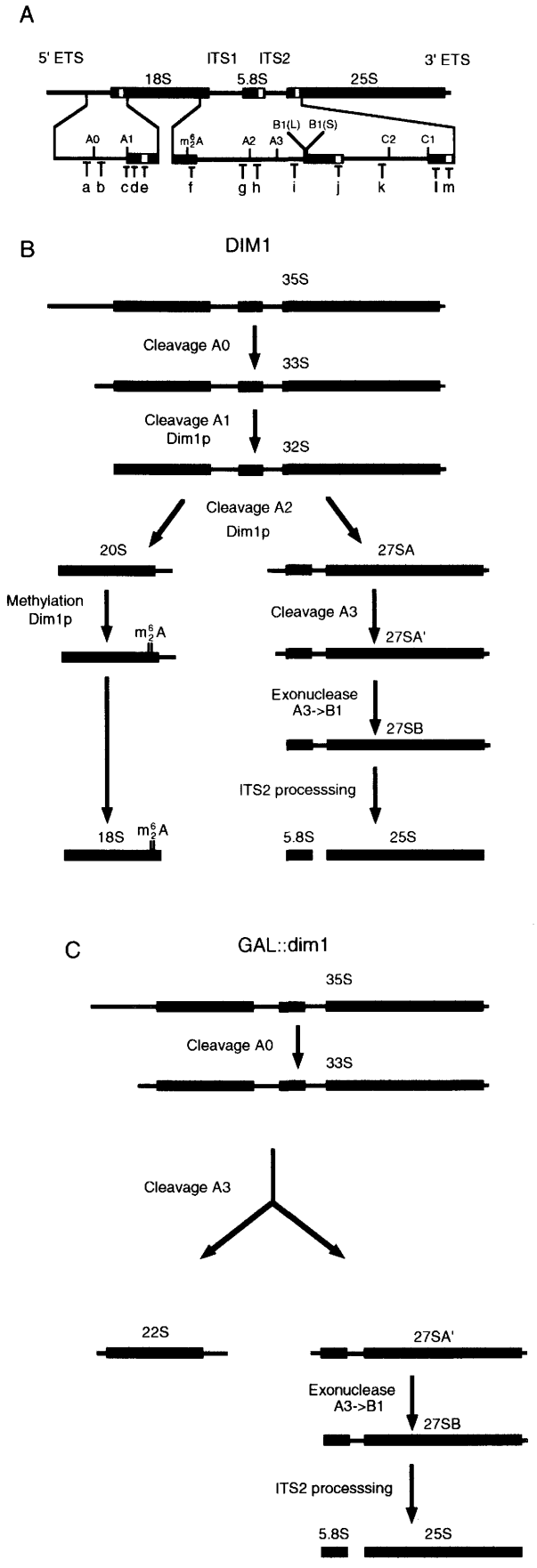

Figure 1. Structure of the yeast pre-rRNA and processing pathway. (*A*) Organization of the 35S pre-rRNA. In the 35S pre-rRNA, the 18S, 5.8S, and 25S rRNA genes are preceded and followed by noncoding sequences, the 5′ and 3′ external transcribed spacers (5′ ETS and 3′ ETS), and separated by internal transcribed spacers 1 and 2 (ITS1 and ITS2). The pre-rRNA cleavages sites are indicated in uppercase, and oligonucleotides used for Northern hybridization and primer extension in lowercase. The dimethylation site is indicated as m_2^6A. (*B*) The yeast pre-rRNA processing pathway. The 35S pre-rRNA is successively cleaved at site A0 in the 5′ ETS, at site A1, the 5′ end of mature 18S rRNA, and at site A2 in ITS1. The first two cleavages yield the 33S and 32S pre-rRNAs, respectively. Cleavage at site A2 splits the 32S precursor into the 20S and 27SA pre-rRNA that contain the RNA components of the small and large ribosomal subunits, respectively. The 20S pre-rRNA is reported to be exported to the cytoplasm where 3′ processing generates the mature 18S rRNA. The 27SA pre-rRNA is processed into 27SA′ pre-rRNA by cleavage at site A3 by RNase MRP. A3 acts as the entry site for an exonuclease activity, which generates the 5′ end of the 27SB(S) pre-rRNA at site B1(S). This site is also the 5′ end of the mature 5.8S(S) rRNA, the major, short form of the 5.8S rRNA. Subsequent cleavages in ITS2 generate the mature 5.8S(S) and 25S rRNAs. For simplicity, only the major processing pathway from 27SA to 5.8S(S) and 25S rRNA is shown; an alternative pathway generates the minor 5.8S(L) rRNA, which is 7–8 nucleotides 5′ extended (for a review of the pre-rRNA processing pathway, see Morrissey and Tollervey 1995). Reactions that require Dim1p are indicated. (*C*) Pre-rRNA processing in the *GAL::dim1* strain. The 35S pre-rRNA is cleaved normally at site A0. The 33S pre-rRNA accumulates and is cleaved at site A3, yielding the 27SA′ pre-rRNA that is processed to 5.8S(S) and 25S and an aberrant 22S pre-rRNA that is not processed to 18S rRNA.

in wild-type yeast cells treated with ethionine, processing of the 35S pre-rRNA still occurs (Tollervey et al. 1993). The *nop1-3* mutation does not, however, inhibit the late methylation of the 20S pre-rRNA at $m_2^6A_{1779}m_2^6A_{1780}$ (Tollervey et al. 1993; D. Tollervey, unpubl.). Finally, yeast *pet56* mutants, which lack a ribose methylation in the mitochondrial large subunit rRNA, are defective in production of the large ribosomal subunit (Sirum-Connolly and Mason 1993).

Although the rRNAs of all organisms are methylated, the precise nucleotides that are modified are not generally conserved from eukaryotes to bacteria. An exception is the $m_2^6A_{1518}m_2^6A_{1519}/m_2^6A_{1779}m_2^6A_{1780}$ sequence (using, respectively, the *Escherichia coli* and the *Saccharomyces cerevisiae* numbering systems), localized in the loop of the hairpin helix 45, at the very 3′ end of the small subunit rRNA. Not only the modification but the whole structure of this region is highly conserved (van Knippenberg et al. 1984). The functional role of the $m_2^6Am_2^6A$ modification has been studied extensively in *E. coli*, (for review, see van Knippenberg 1986) using mutants in *ksgA*, which encodes the dimethylase (van Buul and van Knippenberg 1985); *ksgA* mutants fail to dimethylate the 16S rRNA and, in consequence, are resistant to kasugamycin (an antibiotic related to the aminoglycoside family). The nondimethylated ribosomal

particles are mildly impaired in protein synthesis, mainly at the initiation and elongation steps, and *ksgA* mutants show a slight decrease in growth rate.

In eukaryotes, the functional role of this dimethylation, and indeed all other rRNA modifications, is unknown. The putative yeast 18S rRNA dimethylase gene, *DIM1*, was cloned by complementation of the kasugamycin sensitivity of the *E. coli ksgA* mutant. Dim1p is clearly homologous to KsgAp and can dimethylate the *E. coli* 16S rRNA in vivo (Lafontaine et al. 1994). In contrast to *E. coli ksgA*, yeast *DIM1* is essential for viability, and we therefore generated a conditional allele to determine whether Dim1p is required for ribosome synthesis or function.

Results

Subcellular localization of the dimethylation event

The presence of the $m_2^6Am_2^6A$ methylation can be detected by primer extension because the modification prevents normal base-pairing and therefore blocks reverse transcriptase. In Figure 2 this is shown by primer extension on pre-rRNA transcribed from the pGAL::rDNA plasmid (Henry et al. 1994) that contains the entire pre-rRNA coding region placed under the control of an RNA polymerase II promoter. This was expressed in an *rpa12* mutant strain, which is thermosensitive for RNA Pol I (Nogi et al. 1993), allowing transcription of the chromosomal rDNA to be inhibited. On wild-type pre-rRNA, the methylation can be detected using oligonucleotides that hybridize within the 20S pre-rRNA (e.g., oligonucleotide g; see Fig. 1) but not using oligonucleotides that hybridize further 3′ (e.g., oligonucleotides h–m; see Fig. 1). This is shown for oligonucleotides g and h in Fig. 2 (wild-type lanes); these oligonucleotides hybridize to sequences in ITS1 that are separated only by 13 nucleotides, but cleavage site A2, the 3′ end of the 20S pre-rRNA, lies in this interval. This is consistent with previous reports (Klootwijk et al. 1972; Salim and Maden 1973; Brand et al. 1977) that methylation is a late maturation event occurring on the 20S pre-rRNA prior to its conversion to 18S rRNA. Deletion of the 3′ region of ITS1 can delay pre-rRNA processing and synthesis of 20S pre-rRNA (Henry et al. 1994). On such mutant pre-rRNA, primer extension stops resulting from the $m_2^6Am_2^6A$ methylation can be observed with oligonucleotides h–m (shown for oligonucleotide h in Fig. 2, ITS1Δ3′ lanes), demonstrating that the 32S pre-rRNA, or larger precursors, is methylated. Similarly, mutations in *RRP2* (*NME1*) or *POP1*, which encode the RNA and protein components of RNase MRP, respectively, also delay synthesis of 20S pre-rRNA (Lygerou et al. 1994) and lead to methylation of the 32S pre-rRNA (data not shown). Because the processing of 32S to 20S pre-rRNA is nucleolar, methylation can occur in the nucleolus.

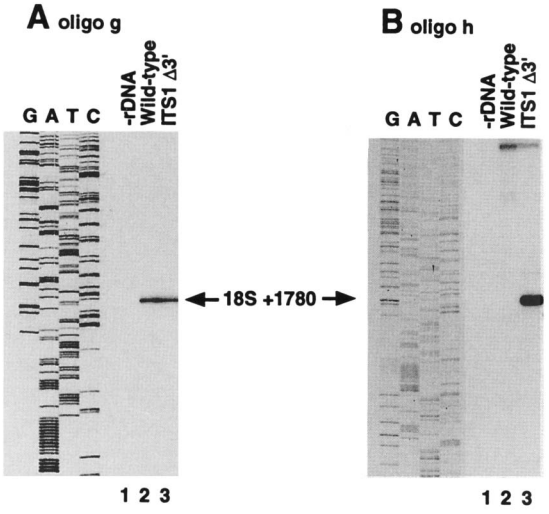

Figure 2. Primer extension through the dimethylation site using oligonucleotides g and h, which hybridize to sequences in ITS1 5′ and 3′ to site A2, respectively (Fig. 1). RNA was extracted from *rpa12* strains carrying pGAL::rDNA containing the wild-type rDNA (wild-type lanes), pGAL::rDNA containing a deletion of the 3′ region of ITS1 (ITS1Δ3′ lanes), or the same vector lacking the rDNA (−rDNA lanes), following growth at 37°C for 6 hr. DNA sequencing reactions on the wild-type rDNA plasmid using the same primers are also shown.

Construction of a conditional DIM1 *allele*

To investigate the function of Dim1p, a conditional allele was created. The *DIM1* promoter was replaced in vitro by the *GAL10* promoter, and the *GAL10::dim1* construct was used to replace the chromosomal *DIM1* gene in a haploid yeast strain (see Materials and methods). Genes under the control of a *GAL* promoter are expressed when the cells are grown on medium containing galactose and repressed by growth on glucose (Johnston and Davis 1984). This allows Dim1p to be depleted from the cells by growth following a shift from galactose to glucose medium. Northern hybridization demonstrated that the level of the *DIM1* mRNA falls rapidly after transfer of the *GAL::dim1* strain to glucose medium (data not shown). Experiments shown in Figures 3, 4, 5, and 7, below, compare the *GAL::dim1* strain with the otherwise isogenic parental *DIM1* strain.

The *GAL::dim1* strain was pregrown on galactose minimal medium to mid-log phase and transferred to glucose minimal medium. For approximately six generations after transfer to glucose, the *GAL::dim1* strain grows identically to the wild-type strain, doubling every 2.5 hr. From 15 hr on, there is a progressive increase in the doubling time of the *GAL::dim1* strain, whereas the wild type maintains a constant doubling time of 2.5 hr. After 33 hr of growth on glucose, the *GAL::dim1* strain has a doubling time of 5.1 hr. Repression of the *GAL::dim1* construct is probably leaky, allowing residual Dim1p synthesis; this is also the case for many other genes expressed under the control of *GAL* promoters.

To test for depletion of the dimethylation activity, primer extension was performed through the site of dimethylation (Fig. 3) using a primer that hybridizes within the 20S pre-rRNA (oligonucleotide g in Fig. 1).

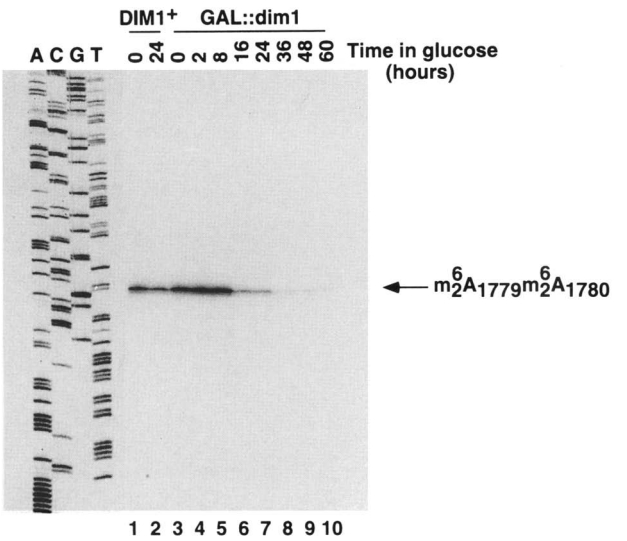

Figure 3. Primer extension through the dimethylation site. Primer extension was performed using oligonucleotide g, which hybridizes to the 20S pre-rRNA within ITS1 (Fig. 1). (Lane *1*) RNA extracted from the wild-type strain following growth on galactose medium; (lane *2*) RNA extracted from the wild-type strain following growth on glucose medium for 24 hr; (lane 3) RNA extracted from the *GAL::dim1* strain following growth on galactose medium; (lanes *4–10*) RNA extracted from the *GAL::dim1* strain following growth on glucose medium for 2, 8, 16, 24, 36, 48, and 60 hr, respectively. RNA was extracted from equal numbers of cells, as judged by OD_{600} units. The positions of primer extension stops resulting from the presence of the modification are indicated. DNA sequencing reactions using oligonucleotide g are also shown.

Total RNA was extracted from equivalent numbers of cells from the wild-type (*DIM1*$^+$) and *GAL::dim1* strains grown on galactose (0 hr) and at intervals after transfer to glucose medium. In the *GAL::dim1* strain, the level of dimethylated pre-rRNA species decreases during growth on glucose medium, the largest drop occurring between 8 and 16 hr following transfer (Fig. 3). These data indicate that Dim1p is responsible for making the $m_2^6A_{1779}m_2^6A_{1780}$ dimethylation.

Even at late time points, the $m_2^6A_{1779}m_2^6A_{1780}$ primer extension stop does not completely disappear, consistent with a lack of complete depletion of Dim1p. The same RNA preparations were used for the experiments shown in Figures 3, 4, 5, and 7A; the data presented below do not indicate that the total level of pre-rRNA species that contain the site of methylation and the site of priming is greatly reduced in strains depleted of Dim1p. The decrease in methylated pre-rRNA is therefore not the result of rapid degradation of the pre-rRNA.

Effects of Dim1p depletion on pre-rRNA processing

The steady-state levels of the mature rRNAs and pre-rRNAs during the time course of Dim1p depletion were analyzed by Northern hybridization (Fig. 4A–F). The level of 18S rRNA falls during depletion, whereas the 25S rRNA level remains constant (Fig. 4A).

The pre-rRNA processing pathway is shown in Figure 1. The 35S pre-rRNA is initially cleaved at site A0 in the 5′ ETS generating the 33S pre-rRNA; the 33S pre-rRNA is then cleaved at site A1, the 5′ end of the mature 18S rRNA sequence, generating the 32S pre-rRNA. The 35S rRNA is detected by all pre-rRNA probes (Fig. 4B–F); the level of the 35S pre-rRNA is little affected in the *GAL::dim1* strain. A probe specific for the 5′ ETS sequence present in 33S but not on 32S (Fig. 4C) reveals that in the *GAL::dim1* strain the 33S pre-rRNA accumulates strongly, commencing 16 hr after transfer to glucose medium. The 33S and 32S pre-rRNAs are very similar in size and are not well resolved by Northern hybridization; however, close inspection reveals the mobility shift indicative of a change from 32S to 33S at 16 hr after transfer to glucose (e.g., see Fig. 4B, lanes 5,6). Comparison of the signal in panels in which both 33S and 32S are detected (Fig. 4B,D–F) with the 33S-specific signal (Fig. 4C) are also consistent with a reduction in the level of 32S from 16 hours after transfer. We conclude that 33S pre-rRNA is accumulated whereas 32S pre-rRNA is depleted and therefore that processing at site A0 is not affected whereas processing at A1 is inhibited.

In the wild type, the 32S pre-rRNA is cleaved at site A2 in ITS1, generating the 20S and 27SA pre-rRNAs. Both the 20S pre-rRNA (Fig. 4D) and 27SA (Fig. 4E,F) pre-rRNAs are underaccumulated in the *GAL::dim1* strain, with the pre-rRNA levels dropping substantially between 8 and 16 hr after transfer to glucose medium. In addition, a novel 22S pre-rRNA species is detected in the *GAL::dim1* strain. This species is detected with probes to the region between A0 and A1 (probes b and c in Fig. 1; shown for probe c in Fig. 4C) and between A2 and A3 (probe h in Fig. 1; Fig. 4E) but is not detected with a probe 5′ to site A0 (probe a in Fig. 1; data not shown) or 3′ to site A3 (probe i in Fig. 1; Fig. 4F). From its size and hybridization pattern, the 22S pre-rRNA is likely to extend from site A0 to site A3. This is the expected product if pre-rRNA processing is inhibited at sites A1 and A2 but continues at sites A0 and A3.

In contrast to the pathway of 18S rRNA synthesis, the 27SB precursor, which is specific to the processing pathway leading to the 5.8S and 25S rRNAs, is not affected by depletion of Dim1p (Fig. 4B). The 27SA′ pre-rRNA cannot be detected by Northern hybridization, but primer extension through site A3, the 5′ end of 27SA′, shows that its accumulation is unaffected in the *GAL::dim1* strain (data not shown). The levels of the 5.8S rRNA and its 7S precursor are also unaffected in the *GAL::dim1* strain, and no alteration was detected in the relative use of the alternative processing pathways that lead to the formation of the long and short forms of 5.8S rRNA (Henry et al. 1994, Lygerou et al. 1994) (data not shown).

Genetic depletion of the RNA or protein components of the U3, U14, snR10, or snR30 small nucleolar ribonucleoproteins (snoRNPs) also inhibits processing at sites A1 and A2, preventing synthesis of the mature 18S rRNA (Tollervey 1987; Li et al. 1990; Hughes and Ares

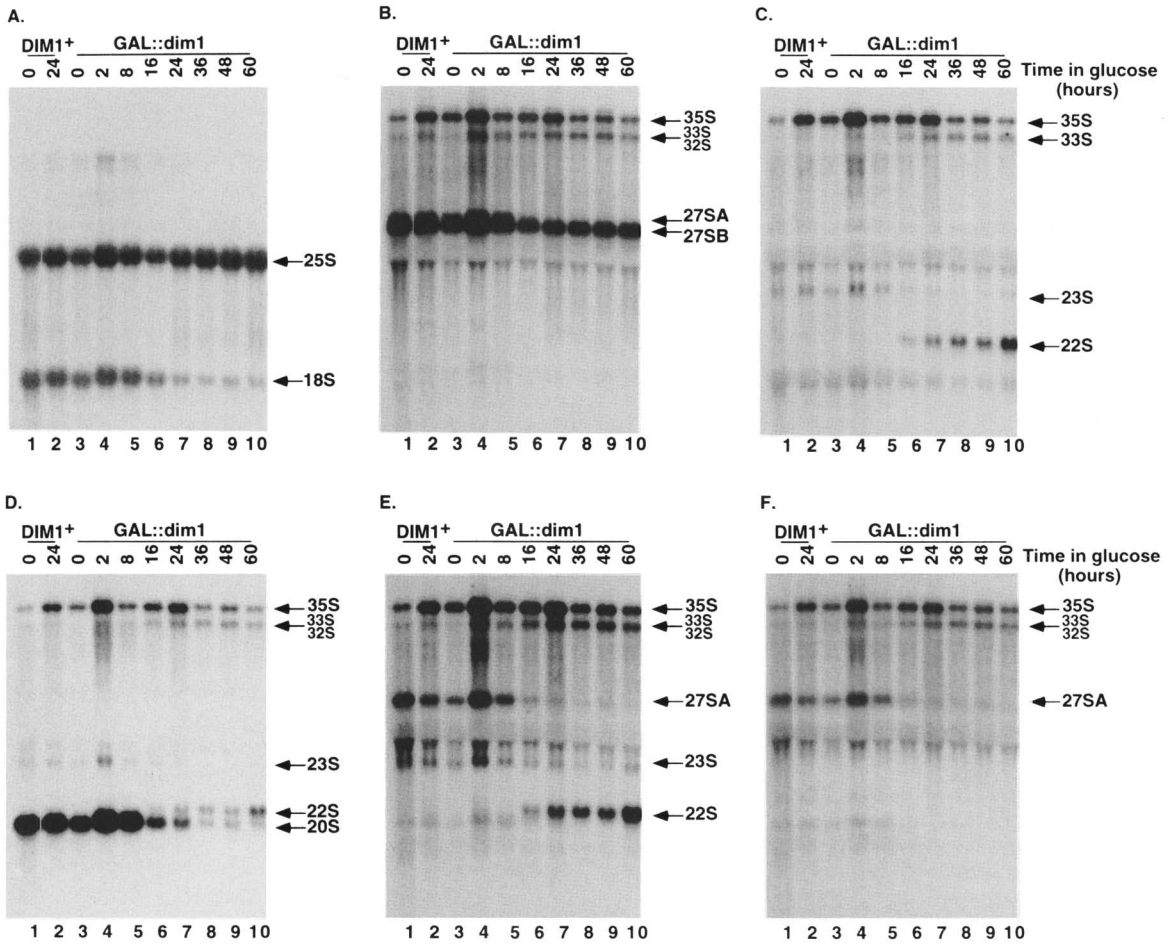

Figure 4. Steady-state levels of mature and precursor rRNA. (Lane *1*) RNA extracted from the wild-type strain following growth on galactose medium; (lane *2*) RNA extracted from the wild-type strain following growth on glucose medium for 24 hr; (lane *3*) RNA extracted from the *GAL::dim1* strain following growth on galactose medium; (lanes *4–10*) RNA extracted from the *GAL::dim1* strain following growth on glucose medium for 2, 8, 16, 24, 36, 48, and 60 hr, respectively. (*A*) Hybridization with oligonucleotides complementary to the mature 18S and 25S rRNAs (oligonucleotides d and l). (*B*) Hybridization with an oligonucleotide complementary to the 5′ region of ITS2 (oligonucleotide k). (*C*) Hybridization with an oligonucleotide complementary to the 3′ region of the 5′ ETS, between cleavage site A0 and A1 (oligonucleotide c). (*D*) Hybridization with an oligonucleotide complementary to the 5′ region of ITS1, upstream of cleavage site A2 (oligonucleotide g). (*E*) Hybridization with an oligonucleotide complementary to the central region of ITS1, between cleavage sites A2 and A3 (oligonucleotide h). (*F*) Hybridization with an oligonucleotide complementary to the 3′ region of ITS1, downstream of cleavage site A3 (oligonucleotide i). RNA was extracted from equal numbers of cells, as judged by OD_{600} units. The positions of the mature rRNAs and the pre-rRNAs are indicated; the 33S and 32S pre-rRNAs are not well resolved. The positions of the oligonucleotides used are indicated in Fig. 1.

1991; Tollervey et al. 1991; Girard et al. 1992; Jansen et al. 1993; Morrissey and Tollervey 1993). The pattern of processing observed in these mutants is, however, different from that seen in the Dim1p-depleted strain; no accumulation of the 33S or 22S pre-rRNAs is observed, instead the snoRNP mutants accumulate a 23S pre-rRNA that extends from the 5′ end of the 35S pre-rRNA to site A3 and is generated by direct cleavage of the 35S pre-rRNA at A3 (Henry et al. 1994). The 23S pre-rRNA can be detected at a low level in wild-type strains (Figs. 4C,D, lanes 1,2) but does not increase in the *GAL::dim1* strain during growth on glucose medium.

The kinetics of the inhibition of pre-rRNA processing and pre-rRNA methylation during depletion of Dim1p are in close agreement; compare the loss of 20S pre-rRNA in Figure 4D with the loss of the primer extension stop resulting from the $m_2^6A_{1779}m_2^6A_{1780}$ modification in Figure 3.

Primer extension was used to determine whether the 33S and 22S pre-rRNA species that accumulate in the *GAL::dim1* strain have 5′ ends at site A0 or are the result of processing at an aberrant site (Fig. 5). Strong accumulation of pre-rRNA species with 5′ ends at site A0 is seen in the *GAL::dim1* strain during growth on glucose medium. The time course of accumulation is in good agreement with the accumulation of the 33S and 22S pre-

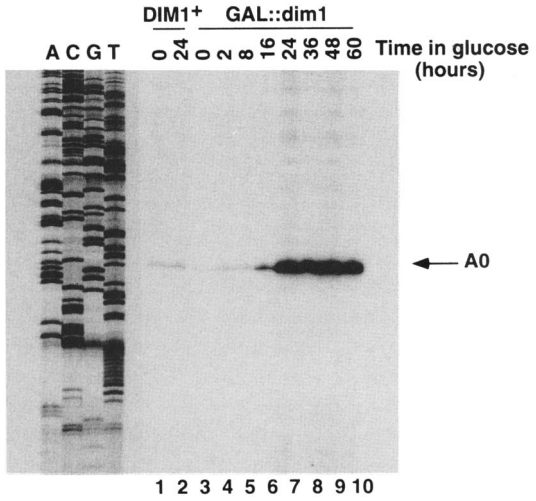

Figure 5. Primer extension in the 5′ ETS. Primer extension was performed using oligonucleotide d, which hybridizes within the 5′ region of the 18S rRNA (Fig. 1). (Lane *1*) RNA extracted from the wild-type strain following growth on galactose medium; (lane *2*) RNA extracted from the wild-type strain following growth on glucose medium for 24 hr; (lane *3*) RNA extracted from the *GAL::dim1* strain following growth on galactose medium; (lanes *4–10*) RNA extracted from the *GAL::dim1* strain following growth on glucose medium for 2, 8, 16, 24, 36, 48, and 60 hr, respectively. The position of the primer extension stop as a result of the cleavage of the pre-rRNA at site A0 is indicated. DNA sequencing reactions using oligonucleotide d are also shown.

rRNAs detected by Northern hybridization. Primer extension also shows that the residual processing at sites A1 and A2 is correct at the nucleotide level at all time points in the *GAL::dim1* strain, as is processing at sites A3, B1(L), and B1(S) (data not shown).

We conclude that in the *GAL::dim1* strain, cleavage of the pre-rRNA is specifically inhibited at sites A1 and A2, the 5′ and 3′ ends of the 20S pre-rRNA, respectively. This would prevent the synthesis of the 20S pre-rRNA and, therefore, of the mature 18S rRNA. Upstream processing of the 35S primary transcript at site A0 in the 5′ ETS is not affected nor are subsequent processing steps on the pathway of synthesis of 5.8S and 25S rRNA.

To confirm that mature 18S rRNA is synthesized with a reduced efficiency in the *GAL::dim1* strain, a metabolic labeling experiment was performed (Fig. 6). Following 25 hr of growth in glucose medium, cells were pulse-labeled for 1 min with [^{3}H]uracil and then chased with a large excess of unlabeled uracil for the times indicated. Total incorporation of [^{3}H]uracil into rRNA in the mutant strain is lower than in the wild type, presumably because of the effects of the reduced growth rate on pre-rRNA transcription. Comparison of the ratio of 18S–25S rRNA synthesis makes it clear, however, that 18S rRNA is underaccumulated in the *GAL::dim1* strain (cf. lanes 4 and 8). Synthesis the of 27SA and 20S pre-rRNAs is also strongly reduced in the *GAL::dim1* strain. The 22S pre-rRNA species is readily detected (Fig. 6, lanes 7,8), demonstrating that it is a major processing product in the Dim1p-depleted strain, rather than being synthesized at low levels and accumulating in the mutant. The residual 18S rRNA that is synthesized in the mutant does not appear to be less stable than in the wild type because it is resistant to chasing for a period of 60 min (data not shown).

Even at late time points after transfer of the *GAL::dim1* strain to glucose medium, the 20S (Fig. 4D) and 27SA (Fig. 4E,F) pre-rRNAs can be detected, showing that some residual processing at sites A1 and A2 continues. The question therefore arises whether this is the result of the processing of the residual methylated pre-rRNA species or whether nonmethylated pre-rRNAs can also be processed, resulting in the synthesis of nonmethylated 18S rRNA. As described above, primer extension through site $A_{1779}A_{1780}$ can be specifically blocked by the presence of the dimethyl groups. This was used to determine whether the residual 18S rRNA which is synthesized in the mutant is dimethylated. To assess the degree of methylation, primer extension was carried out from oligonucleotide f, complementary to the 3′ end of 18S rRNA (Figs. 1 and 7C) using dideoxyadenosine nucleotides in the reaction in place of deoxyadenosine. On nondimethylated rRNA, reverse transcriptase extends through the $A_{1779}A_{1780}$ site and is specifically blocked two nucleotides further 5′, at U_{1777}, owing to the incorporation of dideoxyadenosine into the cDNA. The ratio of readthrough (product c; Fig. 7) to m_2^6A terminated product (products a+b) is a measure of the level of nonmodified versus modified 18S rRNA.

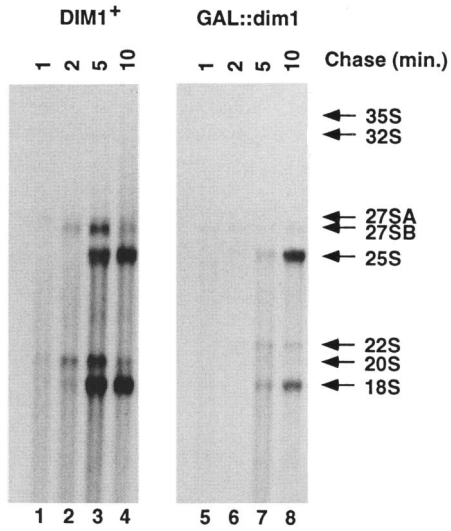

Figure 6. Metabolic labeling of pre-rRNA from wild-type (*DIM1*$^+$) and *GAL::dim1* strains following growth on glucose medium for 25 he. (Lanes *1–4*) RNA extracted from the wild-type strain; (lanes *5–8*) RNA extracted from the *GAL::dim1* strain. Cells were pulse-labeled with [^{3}H]uracil for 1 min at 30°C. A large excess of unlabeled uracil was then added, and the cells were incubated for 1, 2, 5, and 10 min as indicated. The positions of the mature rRNAs and the major pre-rRNAs are indicated.

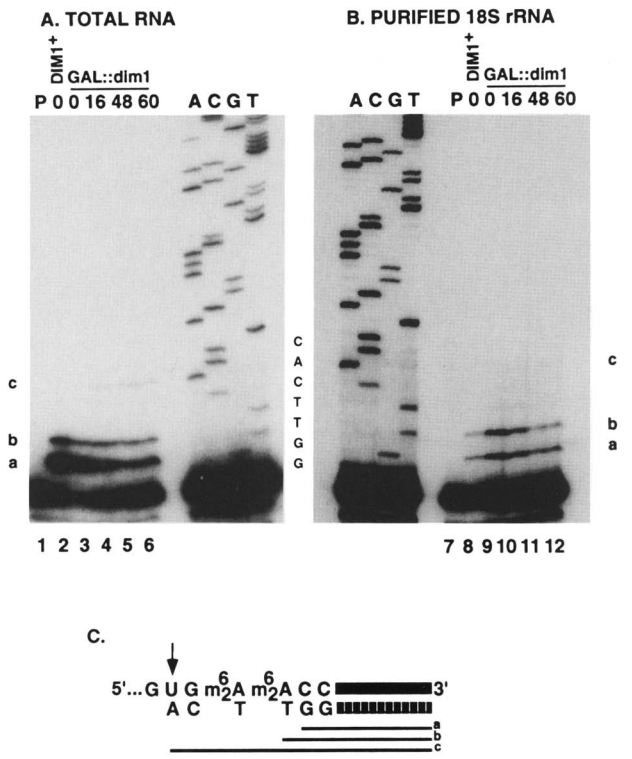

Figure 7. Primer extension through the site of dimethylation. Primer extension was performed with oligonucleotide f, complementary to the 3′ end of 18S rRNA (Fig. 1) and using only dideoxyadenosine nucleotides in place of deoxyadenosine. The site of priming is 3 nucleotides 3′ to A_{1780}, and no A residues are incorporated before the site of modification. Dimethylation of $A_{1779}A_{1780}$ blocks primer extension at that site. Extensions carried out on nondimethylated rRNA extend through the $A_{1779}A_{1780}$ site but are blocked 2 nucleotides 5′ to A_{1779} (at position U_{1777}). (*A*) Total RNA. (*B*) Purified 18S rRNA. The same amount of total RNA or gel-purified 18S rRNA was used for each lane. (Lanes *1,7*) No RNA, symbol P denotes primer alone; (lanes *2,8*) RNA extracted from the wild-type strain following growth on galactose medium; (lanes *3,9*) RNA extracted from the *GAL::dim1* strain following growth on galactose medium; (lanes *4–6,10–12*) RNA extracted from the *GAL::dim1 strain following growth on glucose medium for 16, 48, and 60 hr, respectively*. (*C*) Schematic representation of the 3′ end of 18S rRNA. (Upper line) rRNA strand; the thick line represents the last 16 nucleotides. (Lower line) Complementary cDNA strand; the thick broken line represents oligonucleotide f. The three potential extended products are represented by thin lines (labeled a, b, and c). The positions of the primer extension stops resulting from the presence of the modification are indicated as a and b; the position of the primer extension stop resulting from readthrough of the dimethylation site is indicated as c. DNA sequencing reactions using oligonucleotide f are shown, and the nucleotide sequence, corresponding to the cDNA strand (lower lane of *C*), is indicated between *A* and *B*. It is not clear whether the stop identified as b is a consequence of incomplete dimethylation or is a primer extension artifact.

When this experiment is carried out on standardized amounts of purified 18S rRNA (Fig. 7B), the readthrough is not detected. On total RNA (Fig. 7A) some readthrough is expected owing to newly transcribed pre-rRNA molecules that are still nondimethylated (Fig. 7A, lanes 2,3). Following transfer of the *GAL::dim1* strain to glucose medium, the level of readthrough increases (Fig. 7A, lanes 4–6), presumably as a result of the accumulation of nonmethylated 33S and 22S pre-rRNA, whereas the m_2^6A terminated product declines as a result of the reduced accumulation of mature 18S rRNA.

Nondimethylated 18S rRNA is not detected even 60 hours (>12 generations) after transfer of the *GAL::dim1* strain to glucose medium; if the 18S rRNA synthesized after Dim1p depletion were nondimethylated, it would have been expected to constitute a major fraction of the rRNA by this time. The low level of 18S rRNA observed at late time points by Northern hybridization (Fig. 4A, lanes 8-10) and pulse–chase labeling (Fig. 6, lanes 7,8) can therefore be attributed to processing of residual, methylated 20S pre-rRNA. Although we cannot exclude the possibility that some level of unmethylated 18S rRNA is synthesized and immediately degraded, our data strongly indicates that only correctly methylated pre-rRNA species are processed. This could be due to a requirement for the formation of the $m_2^6Am_2^6A$ residues or to a requirement for an association of Dim1p with the pre-rRNA; the absence of Dim1p binding might prevent both processing and methylation.

Construction of a cis*-acting $G_{1779}G_{1780}$ mutant*

To test whether formation of the dimethyladenines is required for pre-rRNA processing, a pre-rRNA mutation was created in which the twin adenosines $A_{1779}A_{1780}$ were exchanged for guanosine residues. To assess its effect on ribosome biogenesis, the $G_{1779}G_{1780}$ mutation was expressed from plasmid pGAL::rDNA (Henry et al. 1994) in an *rpa12* strain (Nogi et al. 1993). As described above, chromosomal rDNA transcription is inhibited at the nonpermissive temperature (37°C), allowing analysis of the processing of the mutant pre-rRNA. The low level of residual chromosomal transcription does not interfere with the identification of pre-rRNA produced from the pGAL::rDNA plasmid. The presence of neutral tags in the 18S, 5.8S, and 25S rDNA sequences in pGAL::rDNA also allows the identification of mature rRNAs synthesized from the mutant pre-rRNA at all temperatures (Beltrame et al. 1994; Henry et al. 1994).

The absence of modification in the mutant pre-rRNA was confirmed by primer extension (Fig. 8). The guanosine residues should not be a substrate for N^6, N^6 dimethylation, and no methylation of the mutant pre-rRNA is detected. The same RNA preparations were used for the experiments shown in Figures 10 and 11, below, demonstrating that the pre-rRNAs are present, and on longer exposures, a similar level of background primer extension stops is seen in all lanes (data not shown).

Effects of the $G_{1779}G_{1780}$ mutation on rRNA processing

The pGAL::rDNA plasmid carrying the wild-type rDNA

supports growth of the *rpa12* strain at 37°C (Fig. 9, wild-type sectors). In contrast, the same plasmid carrying the rDNA with $G_{1779}G_{1780}$ mutation (Fig. 9, $G_{1779}G_{1780}$ sectors) or lacking the rDNA (Fig. 9, −rDNA sectors) does not support growth of the *rpa12* strain at 37°C. At 25°C, all strains can grow because the chromosomal rDNA is transcribed. We conclude that this mutation prevents the synthesis of functional ribosomes.

The effects of the $G_{1779}G_{1780}$ mutation on the steady-state levels of mature rRNA and of pre-rRNA were analyzed 6 hr after transfer to 37°C. RNA was extracted, run on an agarose–formaldehyde gel, and transferred for Northern hybridization with a set of probes specific for various regions of the 35S pre-rRNA (Fig. 10A–F). No processing defect is seen in the $G_{1779}G_{1780}$ mutant (Fig. 10A–F). No 33S pre-rRNA accumulation is observed, 27SA and 20S pre-rRNA, as well as mature 18S rRNA, are synthesized normally, and the aberrant 22S pre-rRNA observed upon Dim1p depletion is not detected.

Accumulation of 18S rRNA carrying the $G_{1779}G_{1780}$ mutation is partially cold sensitive (Fig. 11). Northern hybridization of RNA extracted from cells grown at 23°C and hybridized with oligonucleotides complementary to the tags in the 18S and 25S rRNAs (oligonucleotides e and m) reveals that the level of 18S rRNA falls drastically, whereas accumulation of 25S rRNA is unaffected, (Fig. 11, cf. lanes 1 and 3). Growth at 18°C gives identical results (data not shown). The $G_{1779}G_{1780}$ mutation may be responsible for an assembly defect, enhanced at low temperature, leading to rapid 18S rRNA degradation. Even at 37°C, the accumulation of 18S rRNA is slightly lower for the $G_{1779}G_{1780}$ mutant than for the wild type (Fig. 10F, cf. lanes 1 and 3). As no processing defect is detected, this is consistent with increased instability of 18S rRNA molecules that are nondimethylated (Fig. 8) and that may be structurally altered by the $G_{1779}G_{1780}$ replacement. Experiments presented for Dim1p depletion in Figures 3–7 were performed on cells grown at 30°C; depletion of Dim1p at 37°C resulted in an identical processing defect (data not shown).

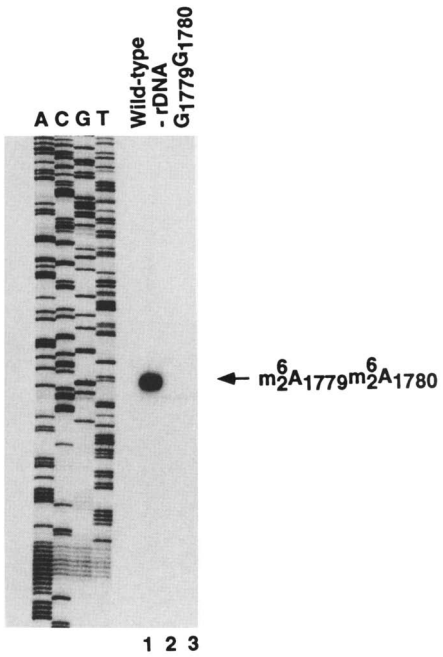

Figure 8. Primer extension through the dimethylation site in the $G_{1779}G_{1780}$ mutant. Primer extension was performed using oligonucleotide g (Fig. 1). (Lane *1*) pGAL::rDNA containing the wild-type rDNA; (lane *2*) the same vector lacking the rDNA sequence; (lane 3) pGAL::rDNA containing the $G_{1779}G_{1780}$ mutation. RNA was extracted from *rpa12* strains carrying the plasmids indicated 6 hr after transfer to 37°C. The same amount of RNA from each strain was used. The positions of primer extension stops resulting from the presence of the modification are indicated. DNA sequencing reactions using oligonucleotide g are also shown.

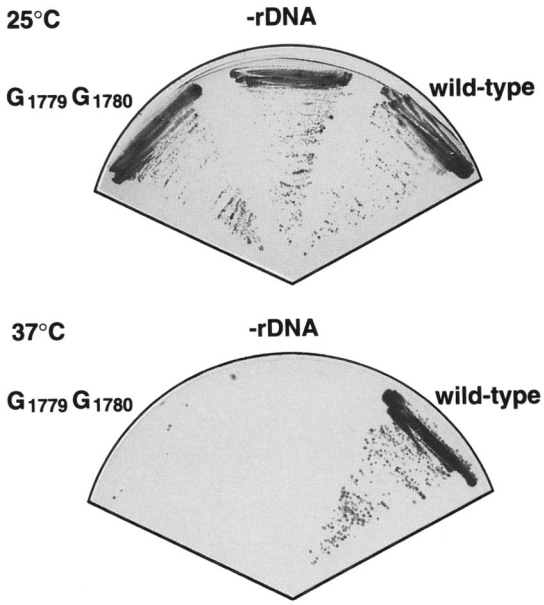

Figure 9. Pre-rRNA carrying the $G_{1779}G_{1780}$ mutation does not support growth. An *rpa12* strain was transformed with pGAL::rDNA containing the wild-type rDNA (wild-type sectors), pGAL::rDNA containing the $G_{1779}G_{1780}$ mutation ($G_{1779}G_{1780}$ sectors), or with the same vector lacking the rDNA sequence (−rDNA sectors). Plates were incubated at 25°C (*top*) or 37°C (*bottom*).

Discussion

DIM1 *is involved in pre-rRNA processing*

The *DIM1* gene was cloned by complementation of a mutation in the *E. coli ksgA* dimethylase, which prevents the 16S rRNA dimethylation. Dim1p is highly homologous to KsgAp and can restore both 16S rRNA dimethylation and kasugamycin sensitivity in *E. coli* (Lafontaine et al. 1994). This made it probable that Dim1p is the enzyme responsible for making the homologous dimethylation, $m_2^6A_{1779}m_2^6A_{1780}$, in the yeast 18S rRNA. We have investigated the function of Dim1p by

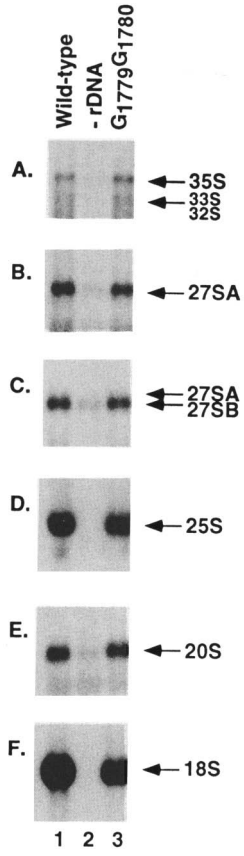

Figure 10. Northern analysis of pre-rRNA and rRNA from the $G_{1779}G_{1780}$ mutant at 37°C. (Lane *1*) pGAL::rDNA containing the wild-type rDNA; (lane *2*) the same vector lacking the rDNA sequence; (lane 3) pGAL::rDNA containing the $G_{1779}G_{1780}$ mutation. RNA was extracted from *rpa12* strains carrying the plasmids indicated 6 hr after transfer to 37°C. The same amount of RNA from each strain was used. (*A,B*) Hybridization with an oligonucleotide complementary to ITS1 between cleavage sites A2 and A3 (oligonucleotide h). (*C*) Hybridization with an oligonucleotide complementary to the 5′ region of ITS2 (oligonucleotide k). (*D*) Hybridization with an oligonucleotide complementary to the 25S rRNA tag (oligonucleotide m). (*E*) Hybridization with an oligonucleotide complementary to the 5′ region of ITS1, upstream of cleavage site A2 (oligonucleotide g). (*F*) Hybridization with an oligonucleotide complementary to the 18S rRNA tag (oligonucleotide e). To simplify the figure, only the relevant regions of each Northern blot are shown; together the six panels present all the pre-rRNAs and rRNAs that we have detected. The positions of the oligonucleotides used are indicated in Fig. 1.

analysis of the effects of growth of a conditional *GAL::dim1* mutant strain on glucose medium. Primer extension shows that the level of dimethylation of the pre-rRNA is progressively reduced during growth of the *GAL::dim1* mutant on glucose medium, confirming that Dim1p is indeed responsible for this modification in yeast.

Analysis of pre-rRNA processing in the *GAL::dim1* strain showed that, unexpectedly, Dim1p is specifically required for the cleavages that generate the 20S pre-rRNA, the immediate precursor to the mature 18S rRNA. Following transfer of the *GAL::dim1* strain to glucose medium, processing is progressively inhibited at site A1, the 5′ end of both the 20S pre-rRNA and the mature 18S rRNA, and at site A2 in ITS1, the 3′ end of the 20S pre-rRNA. In consequence, formation of the 18S rRNA is strongly reduced. In contrast, we detect no requirement for Dim1p in the cleavage of the 5′ ETS at site A0 nor in the subsequent processing reactions that generate the large subunit rRNA species, 5.8S and 25S rRNA. The kinetics of the inhibition of the pre-rRNA processing reactions are in close agreement with the loss of pre-rRNA methylation, suggesting that the processing of nonmethylated pre-rRNA is inhibited.

Even 60 hr (>12 generations) after transfer to glucose medium, we do not detect nonmethylated 18S rRNA in the *GAL::dim1* strain. This shows that the 18S rRNA accumulated in the mutant is processed from the residual methylated pre-rRNA. We conclude that following transfer of the *GAL::dim1* strain to glucose medium there is insufficient Dim1p to methylate all pre-rRNA molecules; only those pre-rRNAs that get methylated are processed at sites A1 and A2. It appears that a system has developed to specifically prevent the synthesis of the 20S pre-rRNA, and therefore the 18S rRNA, in the absence of methylation. We know of no other examples of the regulation of RNA processing reactions in response to modification.

The simplest model for the inhibition of processing would have been that the $m_2^6Am_2^6A$ residues are required for some aspect of ribosome assembly. For example, the correct folding of the (pre)-rRNA or the binding of a ri-

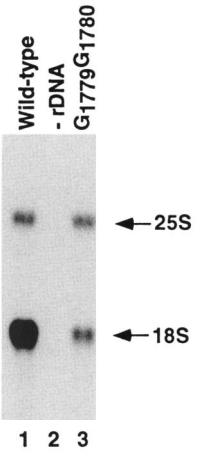

Figure 11. Northern analysis of high-molecular-weight rRNA synthesized from the $G_{1779}G_{1780}$ mutant grown at 23°C. (Lane *1*) pGAL::rDNA containing the wild-type rDNA; (lane *2*) the same vector lacking the rDNA sequence; (lane 3) pGAL::rDNA containing the $G_{1779}G_{1780}$ mutation. RNA was extracted from *rpa12* strains carrying the plasmids indicated following growth at 23°C. The same amount of RNA from each strain was hybridized with oligonucleotides complementary to the tags in the 18S and 25S rRNAs (e and m in Fig. 1).

bosomal protein might have been dependent on $m_2^6Am_2^6A$ formation. The alternative was that some system monitored either the binding of Dim1p itself or the disappearance of the nonmethylated A residues. To distinguish between these possibilities, we constructed and tested a mutation of the pre-rRNA in which both A residues at the site of methylation are replaced by G residues. Processing of the pre-rRNA is unaffected by the double G mutation, demonstrating that the formation of the $m_2^6Am_2^6A$ residues is not required for processing of this pre-rRNA. Moreover, processing of the wild-type pre-rRNA predominantly occurs before the methylation reaction, and formation of the $m_2^6Am_2^6A$ residues presumably cannot, therefore, be required for processing. Together, these results make it most unlikely that formation of $m_2^6Am_2^6A$ is required for processing at sites A1 and A2.

Two general classes of model for the inhibition of pre-rRNA processing in Dim1p-depleted cells can be envisaged. Association of Dim1p with the pre-ribosomal particle might be required for processing; Dim1p might directly interact with components required for processing or be required for correct ribosome assembly. In either case, this could be mediated through the lysine-rich amino-terminal extension of Dim1p that is not present in the *E. coli* KsgAp (Lafontaine et al. 1994). An alternative possibility is that some factor could have a binding site that overlaps with that of Dim1p. Displacement of this factor by Dim1p binding might be required to allow processing. Such a factor could be an RNA or a protein, but it is notable that many of the snoRNA species identified in *S. cerevisiae* are not essential for viability and have no clear growth phenotype when their genes are disrupted. The functions of so many nonessential snoRNAs (10 have been reported to date) are not clear. It might be that some have roles in monitoring the correct assembly or modification of the pre-rRNA. Vertebrate U13 is complementary to the $A_{1779}A_{1780}$ loop region and is, therefore, a potential candidate for such a role. Based on genetic data, cleavage at sites A1 and A2 has been proposed to be coupled and to be carried out by a processing complex that involves multiple snoRNPs (for review, see Fournier and Maxwell 1993; Morrissey and Tollervey 1995). Whatever the actual mechanism involved, this processing complex is a likely target for the coordinated regulation of A1/A2 processing.

Role of the dimethylation in ribosome assembly or function

The two adenosines that are the substrates for dimethylation, are required for ribosomal assembly or function. Ribosomes carrying the $G_{1779}G_{1780}$ mutation do not support growth, showing this mutation to be lethal. Furthermore, the 18S rRNA level drastically falls at 23°C, possibly reflecting an assembly defect, because these are often associated with cold sensitivity. It is not clear at present whether it is the absence of the dimethylation or of the universally conserved adenosine residues at these positions that is responsible for the growth defect and the cold-sensitive phenotype.

Subcellular location of Dim1p and the dimethylation activity

In wild-type yeast strains, dimethylation is not detected on precursors other than the 20S pre-rRNA. However, when synthesis of 20S pre-rRNA is delayed by the inhibition of cleavage at site A2 as a result of the mutation of *cis*- or *trans*-acting factors, dimethylation of the 32S pre-rRNA species is observed. The 32S pre-rRNA is undoubtedly nucleolar, demonstrating that the dimethylation reaction can occur in the nucleolus. We have, moreover, shown here that Dim1p is required for nucleolar pre-rRNA processing reactions. It therefore seems clear that Dim1p can associate with the pre-rRNA in the nucleolus and can function there. On the basis of subcellular fractionation, it has been reported that the 20S pre-rRNA is processed to 18S rRNA in the cytoplasm (Udem and Warner 1973) and that dimethylation occurs on the cytoplasmic 20S pre-rRNA (Klootwijk et al. 1972; Udem and Warner 1973; Brand et al. 1977). It remains possible that in wild-type cells Dim1p is transported to the cytoplasm in association with the 20S pre-rRNA before the dimethylation reaction occurs. The phenomenon of methylation of the 32S pre-rRNA in mutants in which 20S synthesis is delayed suggests that the timing of the actual dimethylation reaction is not dependent on the timing of pre-rRNA processing.

Evolutionary implications

There are a number of similarities between the processing of pre-rRNA in eukaryotes and bacteria. In both, processing on the 5′ and 3′ sides of the small-subunit rRNA is coupled. In bacteria this is achieved by direct base-pairing between the 5′- and 3′-flanking spacer sequences, whereas in eukaryotes, coupling has been proposed to be mediated by interactions in *trans* with the snoRNAs (for review, see by Morrissey and Tollervey 1995). It is possible that the more complex processing system in eukaryotes was, in part, selected because of the greater regulatory potential that it offers. In *E. coli*, inactivation of the KsgAp methylase leads to the synthesis of nonmethylated ribosomes. In contrast, it appears that the association of Dim1p with nucleolar pre-rRNA is monitored in yeast cells by a system that is independent of its dimethylase activity. We postulate that failure of binding activates a regulatory system that inhibits processing and thereby prevents the synthesis of unmodified rRNA. It may have been the potential for just this type of regulatory system that drove selection for the snoRNA-dependent pre-rRNA processing system of eukaryotes.

Materials and methods

Strains and media

Standard *S. cerevisiae* growth and handling techniques were used. Transformation was by the PEG method of Klebe et al.

(1983). The haploid *DIM1*$^+$ strain is BWG1-7A (*MAT***a**, *ura3-52, leu2-3,112, ade1-100, his4-519* and is GAL$^+$) (generously provided by L. Guarente, MIT, Cambridge, MA). The strain used for Dim1p depletion is isogenic except that it has the *URA3–pGAL10::dim1* construct integrated at the *DIM1* locus. Strain BWG1-7A is auxotrophic for uracil; therefore, strain GRF18 (*MAT*α; *leu2-3, 112; his3-11,15, CAN1*) (generously provided by R. Serrano, Seville, Spain) was used for pulse–chase labeling of pre-rRNA with [^{3}H]uracil. The strain used as the host for pGAL:: rDNA expression is NOY504 (*MAT*α; *rpa12::LEU2; leu2-3, 112; ura3-1; trp1-1; his3-11; CAN1-100*) (Nogi et al. 1993; generously provided by M. Nomura, University of California, Irvine, CA).

For Dim1p depletion, cells growing exponentially in galactose minimal medium at 30°C were harvested by centrifugation, washed, and resuspended in glucose minimal medium. During growth, cells were diluted with prewarmed medium and constantly maintained in early exponential phase. Identical results were obtained when the experiment was repeated at 37°C (data not shown).

For analysis of the $G_{1779}G_{1780}$ mutation, strain NOY504 was transformed with plasmid pGAL::rDNA, −rDNA, or pTL2 and grown at 23°C in minimal galactose medium before being transferred, at an OD_{600} of ~0.08 to 37°C for 6 hr. For the experiment presented in Figure 11, RNA was extracted following growth at 23°C; RNA extracted following growth at 18°C gave identical results (data not shown).

Construction of a conditional GAL10::dim1 *allele*

A *Pvu*II–*Xho*I DNA fragment containing the *DIM1* ORF, 5′- and 3′-flanking sequences (Lafontaine et al. 1994) was subcloned into pUC18 to yield plasmid pDL526. In plasmid pDL503, the *GAL1–10* promoter region of plasmid pBM272 (Johnston and Davis 1984; kindly provided by M. Johnston, Washington University School of Medicine, St. Louis, MO), isolated by *Eco*RI–*Bam*HI digestion, was subcloned into plasmid pFL44S (Bonneaud et al. 1991) in order to be fused to a *URA3* marker. The *URA3–pGAL10* cassette was recovered from plasmid pDL503 following a *Cla*I–*Eco*RI digestion, filled-in with the Klenow DNA polymerase, and inserted into plasmid pDL526 at the filled *Cla*I site, which lies 27 nucleotides upstream of the ATG of *DIM1*. The resulting plasmid, pDL527, was digested with *Pvu*II and the fragment, *DIM1* 5′-flanking sequence-*URA3–pGAL10–DIM1* ORF–*DIM1* 3′-flanking sequence, was purified and used to transform yeast strain BWG1-7A, with selection for uracil prototrophy on galactose minimal medium. One URA$^+$ transformant was chosen; integration was checked by PCR and Southern blot analysis (data not shown). All the experiments compared this strain (*GAL::dim1*) with the otherwise isogenic wild-type (*DIM1*$^+$) strain.

Construction of the $G_{1770}G_{1771}$ *mutant*

Construction of plasmids pGAL::rDNA, −rDNA, pITS1Δ3′, and pBSrDNAB-XFTag is described in Henry et al. (1994). The $G_{1779}G_{1780}$ mutation was produced by two-step PCR. The first-round PCR product, using oligonucleotide 5′-TCGCTAGTACCGATTG-3′ and mutagenic oligonucleotide 5′-CCTTCCGCAGGCCCACCTACGG-3′, was used as a primer with oligonucleotide i during the second round of amplification. The second-round amplification product was digested with *Stu*I–*Dra*III and inserted into plasmid pBSrDNAB-XFTag. An *Sfi*I–*Xho*I fragment was recovered from the resulting plasmid pTL1 and inserted into pGAL::rDNA. The final plasmid was termed pTL2.

RNA extraction, Northern hybridization, primer extension, and pulse–chase labeling

RNA was extracted as described previously (Tollervey and Mattaj 1987). Northern hybridization (Tollervey 1987) and primer extension (Beltrame and Tollervey 1992) were as described previously. For the *GAL::dim1* Northern and primer extension experiments, total RNA equivalent to 0.4 OD_{600} of cells (c8×10^6 cells) was used for each sample. For the $G_{1779}G_{1780}$ Northern blots and primer extension experiments, 9 μg of total RNA was used. The primer extension presented in Figure 7B, used ~100 ng of purified 18S rRNA per reaction. 18S rRNA was purified from an agarose–TBE gel using DE81 Whatman chromatographic paper. RNA was eluted for 1 hr at 37°C in 20 mM Tris (pH 7.5), 1 mM EDTA, and 1.5 M NaCl, butanol-extracted, and ethanol-precipitated. The oligonucleotide probes used for Northern hybridization and primer extension are depicted on Figure 1. Oligonucleotide b is GAAAGAAACCGAAATCTC, oligonucleotide c is CCAGATAACTATCTTAAAAG, oligonucleotide f is TAATGATCCTTCCGCA, and oligonucleotide g is CGGTTTTAATTGTCCTA. Oligonucleotides a, h, i, and k are described as B, D, E, and F, respectively, in Morrissey and Tollervey (1993). Oligonucleotides e and m hybridize to the tags in the 18S and 25S rRNAs, respectively, and are described in Beltrame and Tollervey (1992). Oligonucleotide j hybridizes to the tag in the 5.8S rRNA and is described as f in Henry et al. (1994). Oligonucleotides d and l are described as a and g, respectively, in Bergès et al. (1994).

Metabolic labeling of RNA was performed as described previously (Tollervey et al. 1991). Cultures growing exponentially in galactose minimal medium were harvested, washed, and resuspended in prewarmed glucose minimal medium. The cells were subsequently maintained in an early exponential phase by dilution with prewarmed medium. Metabolic labeling with [^{3}H]uracil was carried out as described previously (Tollervey et al. 1991) 25 hr after transfer to glucose medium, except that 7 ml of cells (OD_{600} ~0.3) were labeled with 233 μCi of label and chase times were 1, 2, 5, 10, 20, and 60 min.

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References

Beltrame, M. and D. Tollervey. 1992. Identification and functional analysis of two U3 binding sites on yeast pre-ribosomal RNA. *EMBO J.* **11:** 1531–1542.

Beltrame, M., Y. Henry, and D. Tollervey. 1994. Mutational analysis of an essential binding site for the U3 snoRNA in the 5′ external transcribed spacer of yeast pre-rRNA. *Nucleic Acids Res.* **22:** 5139–5147.

Bergès, T., E. Petfalski, D. Tollervey, and E.C. Hurt. 1994. Synthetic lethality with fibrillarin identifies NOP77p, a nucleolar protein required for pre-rRNA processing and modifica-

tion. *EMBO J.* **13:** 3136–3148.

Bonneaud, N., O. Ozier-Kalogeropoulos, G. Li, M. Labouesse, L. Minvielle-Sebastia, and F. Lacroute. 1991. A family of low and high copy replicative, integrative and single-stranded *S. cerevisiae/E. coli* shuttle vectors. *Yeast* **7:** 609–615.

Brand, R.C., J. Klootwijk, T.J.M. van Steenbergen, A.J. de Kok, and R.J. Planta. 1977. Secondary methylation of yeast ribosomal precursor RNA. *Eur. J. Biochem.* **75:** 311–318.

Brimacombe, R., P. Mitchell, M. Osswald, K. Stade, and D. Bochkariov. 1993. Clustering of modified nucleotides at the functional center of bacterial ribosomal RNA. *FASEB J.* **7:** 161–167.

Caboche, M. and J.-P. Bachellerie. 1977. RNA methylation and control of eukaryotic RNA biosynthesis. *Eur. J. Biochem.* **74:** 19–29.

Fournier, M.J. and E.S. Maxwell. 1993. The nucleolar snRNAs: Catching up with the spliceosomal snRNAs. *Trends Biol. Sci.* **18:** 131–135.

Girard, J.P., H. Lehtonen, M. Caizergues-Ferrer, F. Amalric, D. Tollervey, and B. Lapeyre. 1992. GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast. *EMBO J.* **11:** 673–682.

Henry, Y., H. Wood, J.P. Morrissey, E. Petfalski, S. Kearsey, and D. Tollervey. 1994. The 5′ end of yeast 5.8S rRNA is generated by exonucleases from an upstream cleavage site. *EMBO J.* **13:** 2452–2463.

Hughes, J.M.X. and M.J. Ares. 1991. Depletion of U3 small nucleolar RNA inhibits cleavage in the 5′ external transcribed spacer of yeast pre-ribosomal RNA and impairs formation of 18S ribosomal RNA. *EMBO J.* **10:** 4231–4239.

Jansen, R., D. Tollervey, and E.C. Hurt. 1993. A U3 snoRNP protein with homology to splicing factor PRP4 and Gβ domains is required for ribosomal RNA processing. *EMBO J.* **12:** 2549–2558.

Johnston, M. and R.W. Davis. 1984. Sequences that regulate the divergent *GAL1–GAL10* promoter in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **4:** 1440–1448.

Klebe, R.J., J.V. Harriss, D. Sharp, and M.G. Douglas. 1983. A general method for poly-ethylene-glycol-induced genetic transformation of bacteria and yeast. *Gene* **25:** 333–341.

Klootwijk, J. and R.J. Planta. 1989. Isolation and characterization of yeast ribosomal RNA precursors and preribosomes. *Methods Enzymol.* **180:** 96–109.

Klootwijk, J., R.C. van den Bos, and R.J. Planta. 1972. Secondary methylation of yeast ribosomal RNA. *FEBS lett.* **27:** 102–106.

Lafontaine, D., J. Delcour, A.-L. Glasser, J. Desgrès, and J. Vandenhaute. 1994. The DIM1 gene responsible for the conserved $m_2^6Am_2^6A$ dimethylation in the 3′ terminal loop of 18S rRNA is essential in yeast. *J. Mol. Biol.* **241:** 492–497.

Li, H.V., J. Zagorski, and M.J. Fournier. 1990. Depletion of U14 small nuclear RNA (snR128) disrupts production of 18S rRNA in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **10:** 1145–1152.

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

Maden, B.E.H. 1990. The numerous modified nucleotides in eukaryotic ribosomal RNA. *Prog. Nucleic Acid Res. Mol. Biol.* **39:** 241–303.

Morrissey, J.P. and D. Tollervey. 1993. Yeast snR30 is a small nucleolar RNA required for 18S rRNA synthesis. *Mol. Cell. Biol.* **13:** 2469–2477.

———. 1995. Birth of the snoRNPs–The evolution of RNase MRP and the eukaryotic pre-rRNA processing system. *Trends Biol. Sci.* **20:** 78–82.

Nogi, Y., R. Yano, J. Dodd, C. Carles, and M. Nomura. 1993. Gene *RRN4* in *Saccharomyces cerevisiae* encodes the A12.2 subunit of RNA polymerase I and is essential only at high temperatures. *Mol. Cell. Biol.* **13:** 114–122.

Raué, H.A. and R.J. Planta. 1991. Ribosome biogenesis in yeast. *Prog. Nucleic Acid Res. Mol. Biol.* **41:** 89–129.

Salim, M. and B. Maden. 1973. Early and late methylations in HeLa cell ribosome maturation. *Nature* **244:** 334–336.

Sirum-Connolly, K. and T.L. Mason. 1993. Functional requirement of a site-specific ribose methylation in ribosomal RNA. *Science* **262:** 1886–1889.

Swann, P.F., A.C. Peacock, and S. Bunting. 1975. Carcinogenesis and cellular injury: The effect of ethionine on ribonucleic acid synthesis in rat liver. *Biochem. J.* **150:** 335–344.

Tollervey, D. 1987. A yeast small nuclear RNA is required for normal processing of pre-ribosomal RNA. *EMBO J.* **6:** 4169–4175.

Tollervey, D. and I.W. Mattaj. 1987. Fungal small nuclear ribonucleoproteins share properties with plant and vertebrate U-snRNPs. *EMBO J.* **6:** 469–476.

Tollervey, D., H. Lehtonen, M. Carmo-Fonseca, and E.C. Hurt. 1991. The small nucleolar RNP protein NOP1 (fibrillarin) is required for pre-rRNA processing in yeast. *EMBO J.* **10:** 573–583.

Tollervey, D., H. Lehtonen, R. Jansen, H. Kern, and E.C. Hurt. 1993. Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA methylation, and ribosome assembly. *Cell* **72:** 443–457.

Udem, S.A. and J.R. Warner. 1973. The cytoplasmic maturation of a ribosomal precursor ribonucleic acid in yeast. *J. Biol. Chem.* **248:** 1412–1416.

van Buul, C.P.J.J. and P.H. van Knippenberg. 1985. Nucleotide sequence of the *ksgA* gene of *E. coli*: Comparison of methyltransferases affecting dimethylation of adenosine in ribosomal RNA. *Gene* **38:** 65–72.

van Knippenberg, P.H. 1986. Structural and functional aspects of the N^6,N^6 dimethyladenosines in 16S ribosomal RNA. In *Structure, function and genetics of ribosomes* (ed. B. Hardesty and G. Kramer), pp. 412–424. Springer-Verlag, New York.

van Knippenberg, P.H., J.M.A. van Kimmenade, and H.A. Heus. 1984. Phylogeny of the conserved 3′ terminal structure of the RNA of small ribosomal subunits. *Nucleic Acids Res.* **12:** 2595–2604.

Vaughan, M.H., R. Soeiro, J.R. Warner, and J.E.J. Darnell. 1967. The effects of methionine deprivation on ribosome synthesis in HeLa cells. *Proc. Natl. Acad. Sci.* **58:** 1527–1534.

Warner, J.R. 1989. Synthesis of ribosomes in *Saccharomyces cerevisae. Microbiol. Rev.* **53:** 256–271.

Wolf, S.F. and D. Schlessinger. 1977. Nuclear metabolism of ribosomal RNA in growing, methionine-limited, and ethionine-treated HeLa cells. *Biochem.* **16:** 2783–2791.

Woolford, J.L., Jr. 1991. The structure and biogenesis of yeast ribosomes. *Adv. Genet.* **29:** 63–118.

Woolford, J.L., Jr. and J.R. Warner. 1991. The ribosome and its synthesis. In *The molecular and cellular biology of the yeast* Saccharomyces*: Genome dynamics, protein synthesis and energetics* (ed. J.R. Broach, J.R. Pringle, and E.W. Jones), pp. 587–626. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.