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Molecular genetics of the peptidyl transferase center and the unusual Var1 protein in yeast mitochondrial ribosomes

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Abstract. Mitochondria possess their own ribosomes responsible for the synthesis of a small number of proteins encoded by the mitochondrial genome. In yeast, *Saccharomyces cerevisiae*, the two ribosomal RNAs and a single ribosomal protein, Var1, are products of mitochondrial genes, and the remaining approximately 80 ribosomal proteins are encoded in the nucleus. The mitochondrial translation system is dispensable in yeast, providing an excellent experimental model for the molecular genetic analysis of the fundamental properties of ribosomes in general as well as adaptations required for the specialized role of ribosomes in mitochondria. Recent studies of the peptidyl transferase center, one of the most highly conserved functional centers of the ribosome, and the Var1 protein, an unusual yet essential protein in the small ribosomal subunit, have provided new insight into conserved and divergent features of the mitochondrial ribosome.
Key words. *Saccharomyces cerevisiae*; mitochondrial ribosomes; peptidyl transferase; Var1 ribosomal protein; gene relocation; posttranscriptional rRNA modification.

Introduction

The ribosome is the site of protein synthesis in all cells, and despite the diversity of prokaryotic and eukaryotic organisms, the fundamental features, if not the details, of ribosome structure and function have been highly conserved in evolution. Ribosomes in prokaryotes are responsible for the synthesis of the entire complement of cellular proteins, whereas eukaryotic cells use different translation systems for protein synthesis in the cytoplasm, chloroplasts, and mitochondria. Cytoplasmic ribosomes produce the vast majority of cellular proteins while the ribosomes in chloroplasts and mitochondria only produce the small subset of proteins encoded in the organellar genomes. Mitochondrial ribosomes typically have a very limited role. For example, the mitochondrial ribosomes in the budding yeast *Saccharomyces cerevisiae* produce only seven subunits of the energy-transducing enzyme complexes in the inner mitochondrial membrane and the Var1 protein (Var1p), an essential component of the mitochondrial small ribosomal subunit. These proteins are essential for aerobic energy metabolism but not for cell viability in the presence of fermentable sugars, although for unknown reasons mitochondrial translation is required to maintain the integrity of the mitochondrial genome [1].

The formation of yeast mitochondrial ribosomes requires the expression of both mitochondrial and nuclear genes [2–5]. The contributions from mtDNA are the large (21S) and small (15S) rRNAs and Var1p. The nuclear genes specify approximately 80 ribosomal proteins (r-proteins), three rRNA nucleotide modification enzymes, and an unknown number of proteins required for rRNA processing. Together, these nuclear genes make up approximately 1.5% of the estimated 6,000 genes in the yeast genome. Thus, the cell makes a substantial investment in the formation of ribosomes for the synthesis of only a handful of proteins.

Interest in the yeast mitochondrial ribosome has focused on three main areas. First, the regulatory properties of the genes for the ribosomal components provide information about the coordination of nuclear-mitochondrial interactions in the formation of the mitochondrial translational machinery. Second, the mitochondrial ribosome interacts with nuclear-encoded, mRNA-specific translation factors that are dominant elements in controlling the expression of mitochondrial genes (see review by T. D. Fox in this issue). Finally, information about the conserved and divergent features of the mitochondrial ribosome is relevant to ribosomes in general, including the prototype ribosome in *E. coli*. These areas have been broadly covered in several recent reviews [2–6]. The scope of this review is limited to work from our laboratory on two specific aspects of the yeast mitochondrial ribosome, 1) the petidyl transferase center (PTC), one of the most highly conserved functional centers of the ribosome, and 2) the unusual Var1 r-protein.

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Essential features of the peptidyl transferase center in the yeast mitochondrial ribosome

What is the peptidyl transferase?

The formation of a peptide bond between the amino-acyl-tRNA bound in the A site and the peptidyl-tRNA in the P site is catalyzed by peptidyl transferase, an intrinsic activity of the large ribosomal subunit. An important objective in the field of ribosome research is to understand the structure and catalytic mechanism of the PTC. A large effort has been devoted to defining the minimal requirements for the formation and function of the PTC and identifying the components that participate directly in catalysis. In vitro experiments using bacterial ribosomes, primarily from *E. coli*, have provided much of what is known about the PTC (for recent reviews, see [7–10]). Both the 23S rRNA, principally Domain IV and Domain V, and several r-proteins, including L2, L3, L4, L15, L16 and L27, have been implicated in the catalytically active complex. Neither the rRNA alone nor any of the individual r-proteins have been shown to catalyze the petidyl transferase reaction. Current thinking favors a structural role for the r-proteins in maintaining the conformation of active sites in the catalytic center of the rRNA, although a catalytic role for r-proteins has not been ruled out completely.

The recent emphasis on the catalytic role of the 23S rRNA stems from the discovery of RNA enzymes (ribozymes) and a large body of experimental evidence indicating an intimate involvement of the rRNA in the peptidyl transferase reaction. Perhaps the most persuasive evidence is the demonstration that the peptidyl transferase activity of ribosomes from the thermophilic eubacterium *Thermus aquaticus* is highly sensitive to digestion with ribonuclease but remarkably resistant to treatment with proteinase K, SDS, and vigorous extraction with phenol [11]. Although this extraction procedure removes most of the r-proteins, procedures that remove the residual protein cause unfolding of the RNA and inactivation of the PTC [8]. So far, it has not been possible to obtain peptidyl transferase activity with protein-free in vitro transcripts of 23S rRNA, consequently the question remains open whether peptidyl transferase activity is catalyzed by the rRNA alone or a combination of the rRNA and one or more r-proteins.

Proteins implicated in the PTC of the yeast mitochondrial ribosome

There has been substantial recent progress in the identification and characterization of the nuclear genes for the estimated 40 or more different r-proteins in the mitochondrial large 54S subunit [2]. The current Yeast Protein Database [12] lists 30 confirmed or probable constituents of the 54S subunit, 17 of which bear significant sequence relatedness to bacterial r-proteins (table 1). Significantly, most of the *E. coli* r-proteins implicated in the PTC have yeast mitochondrial homologues, including L2, L3, L4, L15, L16, L23 and L27. Three proteins, L2, L16, and L27, are particularly interesting because crosslinking experiments consistently show that they make contacts with antibiotics that inhibit peptidyl transferase and with the 3′ ends of tRNA bound in the A or P sites (for review, see [10]). The identities of the nuclear genes for the yeast mitochondrial r-proteins related to bacterial L2, L16, and L27 have been confirmed by gene disruption and each protein is essential for ribosomal function in vivo [13–15] (C. Pan and T. L. Mason, unpublished observation).

The lack of a standard nomenclature for yeast mitochondrial r-proteins is a potential source of confusion in the following discussion of the molecular genetic analysis of the mitochondrial L2 and L27 proteins. For example, *MRPL2* and *MRP7* are synonymous names for the gene specifying the mitochondrial L27-like protein, which is called YmL2 or Mrp7p. In the case of the mitochondrial L2 homologue, the names for gene and protein used in the Yeast Protein Database are *YEL050c* and Yel050p, respectively. To minimize confusion and to emphasize the relationships between bacterial and mitochondrial r-proteins, we will refer to the yeast mitochondrial L2- and L27-like proteins/genes as Rml2p/*RML2* and Rml27p/*RML27*, repectively, for Ribosomal Mitochondrial protein Large with the number indicating the related eubacterial r-protein (this designation follows the nomenclature for yeast mitochondrial r-proteins suggested by B. Baum, personal communication).

Table 1. Confirmed and probable yeast mitochondrial ribosomal proteins in the large subunit that have bacterial homologues.

YPD name [12]	N-terminal fragment [12]	Homologue	Genbank accession
YD9727.11		L1	Z48758 × 11
YELO50c		L2	U18779 × 4
MRPL9	YmL9	L3	Z73004 × 2
MRP_575694	YmL6	L4	Z46659 × 14
YD8419.04	YmL7	L5	Z49701 × 04
MRPL6		L6	U10397 × 5
YNL185c		L11	Z71461 × 1
YGL068w		L7/L12	Z72591 × 2
MRPL38	YmL38	L14	Z28169 × 1
YNL284c	YmL10 (int. seq.?)	L15	Z71560 × 3
MRPL16	YmL18 (int. seq.)	L16	Z35799 × 1
MRPL8	YmL8	L17	Z49338 × 1
MRP20	YmL41	L23	U32274 × 23
MRPL2	YmL2	L27	Z71281 × 1
MRPL33	YmL33	L30	Z49704 × 12
MRPL39	YmL39	L33	Z49810 × 9
YD9727.10		L34	Z48758 × 10

Functional role of Rml2p

The large L2 protein family has members in ribosomes from eubacteria, archaebacteria, and the cytoplasm and organelles of eukaryotes. L2 is a primary RNA binding protein in bacteria, and its binding site in Domain IV of the 23S rRNA has been characterized in detail by chemical and ribonuclease footprinting [16]. Moreover, L2 is considered one of the primary candidates for a protein with catalytic activity in the peptidyl transferase reaction [10, 17]. One proposal is that a histidine in L2 participates in chemical catalysis similar to the proteolysis mechanism of serine proteases. Of the nine histidine residues in *E. coli* L2, His-229 is the most highly conserved, occurring at the equivalent position in the over 50 known L2 proteins. The single exception is the possible substitution of glutamine at the position corresponding to His-229 in the predicted L2 protein from *Mycoplasma capricolum*. It should be noted, however, that a histidine is predicted at this position in the closely related species *Mycoplasma gentilium*. Therefore, if a histidine in L2 is involved in catalysis, it is most probably His-229.

E. coli L2 is an essential protein in in vitro reconstitution experiments, and the analysis of L2 variants created by in vitro mutagenesis have confirmed its essentiality in vivo [18]. The mutations targeted the higher conserved sequence Gly-221 to His-231 and included a single substitution of His-229 through Gln (H229Q-L2) and a seven-amino-acid deletion Thr-222-Asp-228 (Δ7-L2). When the L2 variants were overexpressed from a plasmid in cells expressing wild-type L2 from the chromosomal gene, the cells were unable to grow at 37 °C, i.e., overexpression of the mutant proteins had a dominant lethal effect. These results highlight the functional importance of the highly conserved sequence near His-229.

Recently, the functional properties of the H229Q-L2 variant were examined in in vitro reconstitution experiments [17]. The 50S subunits reconstituted with H229Q-L2 appeared identical to subunits reconstituted with wild-type L2 with respect to overall protein composition, the interaction of L2 with 23S rRNA, and the ability to combine with 30S subunits to form 70S ribosomes. Significantly, however, the 50S subunits containing H229Q-L2 were completely inactive in peptidyl transferase activity, consistent with an essential role for His-229 in the peptidyl transferase catalytic center.

Molecular genetic studies of yeast Rml2p confirm the overall importance of this protein in the ribosome, but do not support the idea that His-229 is directly involved in catalysis [18] (C. Pan and T. L. Mason, unpublished observation). Null mutants lacking Rml2p are respiration-deficient and convert to [*rho*$^-$] or [*rho*^{0}] cytoplasmic petites, indicating that Rml2p is essential for mitochondrial translation. Rml2p variants equivalent to two of those studied in *E. coli* L2 were created by substitution of His-343 to Gln (*rml2-H343-Q*) at the position equivalent to His-229 and deletion of the adjacent seven amino acids, Val-336 to Asp-342 (*rml2-Δ7*). Cells that express only the mutant alleles were created by 'plasmid shuffle'. Briefly, low copy number centromere plasmids bearing either *rml2-H343-Q* or *rml2-Δ7* were transformed into a haploid *rml2* null mutant in which wild-type Rml2p is expressed from an episomal *URA3*-containing plasmid. The mutant phenotypes were revealed after subsequent eviction of the *URA3*-containing plasmid.

Deletion of the seven amino-acid sequence from Val-336 to Asp-342 inactivated Rml2p, causing conversion of the mutant to [*rho*$^-$] or [*rho*^{0}]. By contrast, the *rml2-H343-Q* mutant had only a conditional cold-sensitive respiratory deficiency; respiratory growth was normal at the permissive temperature (30 °C) and severely impaired at 18 °C, yet the cells remained [*rho*$^+$]. At the resolution of ribosomal subunit profiles in sucrose gradient analysis, assembly of 54S subunit was normal in cells grown at either 30 °C or 18 °C. Since the *rml2-H343-Q* mutant has normal mitochondrial translation when grown at the permissive temperature, His-343 is clearly not essential for the chemistry of the peptidyl transferase reaction in yeast mitochondria. Given that the mechanism of peptide bond formation is shared by all ribosomes, and His-343 is the most highly conserved histidine in the L2 family, it seems improbable that peptidyl transferase involves a direct catalytic role for a histidine in the L2 proteins.

Structure-function analysis of Rml27p (Mrp7p, Mrl2p)

RML27 (MRP7, MRPL2) encodes a 371 amino-acid-long polypeptide; residues 1 to 27 constitute the mitochondrial targeting presequence, the 84 residues between 28 and 111 align with the 84 residues of the *E. coli* ribosomal protein L27, and the 260 residues at the carboxy-terminus show no significant relatedness to other known proteins [14]. Interest in Rml27p stems from the central location of its homologue L27 in the peptidyl transferase center of the *E. coli* ribosome. Most notably, tRNA-protein cross-linking experiments have shown that L27 makes close contacts with the 3′-ends of both A-site and P-site tRNAs [19, 20]. L27 also crosslinks to L16 [21, 22] and to L33 [23]. Although L27 is not a primary RNA binding protein [24], it crosslinks to three sites in Domain V of 23S RNA [25, 26, 27].

Despite its central location in the PTC, L27 is not an essential r-protein in *E. coli*. Reconstituted particles lacking L27 are capable of peptidyl transferase activity, although at greatly reduced rates [28]. A mutant lacking L27 was reported to grow slowly at 37 °C and not at all at 22 °C [29], and recently this phenotype was confirmed with a knockout allele (*ΔrpmA*) created by replacement of the gene for L27 with a

kanamycin-restance marker [19]. Sucrose gradient centrifugation of ribosomal subunits from the *ΔrpmA* mutant showed a deficiency of 50S subunits and an accumulation of a slowly sedimenting large subunit particle, presumably a precursor to the 50S subunit. In addition, the 50S particles lacking L27 have significantly reduced peptidyl transferase activity. Thus, the lack of L27 impairs the assembly of 50S subunits as well as their function in peptide bond formation.

The unusual structure of Rml27p led us to propose that its short L27-like domain serves a conserved function in the peptidyl transferase center and the nonconserved carboxy-terminal domain provides a function unique to the mitochondrial ribosome [13, 30]. Moreover, the composite nature of this protein suggests that it might have originated from a fusion between smaller genes encoding proteins with separate functions. The relative functional importance of the conserved and nonconserved sequence domains in Rml27p has been evaluated through extensive in vitro mutagenesis. Over fifty *rml27* mutant alleles have been examined for effects on respiratory growth and, in selected cases, on ribosome function and assembly (C. Pan and T. L. Mason, unpublished observation) [13, 18]. The following are the major conclusions from this study:

1. Considering that L27 is not essential in *E. coli*, it is surprising that Rml27p is absolutely essential in the yeast mitochondrial ribosome, and that both the conserved and nonconserved domains are required.
2. Deletions that truncate the coding sequence from the 3′ end as well as single codon changes at each of three universally conserved amino acids in the L27-like domain caused conditional (cold- and/or heat-sensitive) defects in the assembly of the 54S large ribosomal subunit. Although single amino acid substitution and C-terminal truncation mutations caused defective assembly of the large ribosomal subunit under different conditions and to different degrees, each mutant had reduced amounts of mature 54S subunits and each accumulated a defective slowly sedimenting particle. The fact that single amino acid substitutions in the N-terminal domain and C-terminal truncations both affect ribosome assembly underscores the importance of both domains in the assembly process.
3. While the C-terminal sequence is clearly essential as a whole, none of the mutations identify a smaller subsequence that is absolutely required for the formation of functional mitochondrial ribosomes. Interestingly, some relatively small in-frame internal deletions within the carboxy-terminal domain have severe phenotypes whereas larger deletions spanning the same regions are relatively innocuous.
4. When expressed as separate mitochondria-targeted polypeptides, both domains of Rml27p associate independently with the large ribosomal subunit, but the separate polypeptides do not provide function in trans. Therefore, if the two-domain structure of Rml27p resulted from a gene fusion, it is not readily reversed in the laboratory. It is noteworthy that a different result was obtained in a similar experiment with the yeast mitochondrial S15 homologue MRPS28. Huff et al. [31] showed that two inactive fragments of the protein, an amino-terminal nonconserved sequence domain of 117 amino acids and a carboxy-terminal fragment containing the conserved 89 amino acid S15-like domain, can function in trans to support ribosome assembly and respiratory growth.

Taken together, the results of our mutational analysis of Rml7p suggest that the function of the L27-like domain is sensitive to minor perturbations in structure, whereas the structural requirements of the carboxy-terminal domain appear to be much less stringent, yet the two domains are functional only when connected in a single polypeptide chain.

How does an extremely highly conserved functional center in the mitochondrial ribosome accommodate the additional mass of Rml27p? One possibility is that the C-terminal domain of Rml27p is filling the structural and/or functional role of a missing ribosomal component. For example, yeast mitochondrial large subunits lack 5S rRNA, and we have hypothesized that one of the three expansion segments in Domain V of 21S rRNA (fig. 1) might be an embedded equivalent of 5S rRNA [32]. Although more recent comparative sequence data do not support this idea [33], it is interesting that the expansion segments in 21S rRNA are in close proximity to sites where L27 crosslinks to *E. coli* 23S rRNA, and 5S rRNA also crosslinks in Domain V of 23S rRNA (fig. 1) [34, 35]. Three *E. coli* r-proteins, L5, L18 and L25, bind to 5S rRNA [18, 36]. Despite the lack of free-standing 5S rRNA in yeast mitochondria, there is a probable mitochondrial r-protein homologous to *E. coli* L5 (table 1). L5 and L18 are well-conserved in evolution, but there does not appear to be a yeast mitochondrial r-protein homologous to L18. Together, these observations support speculation that the carboxy-terminal end of Rml27p might interact with the putative mitochondrial L5 homologue and one or more of the expansion segments to provide a surrogate for the 5S ribonucleoprotein complex typically found in other ribosomes.

Functional significance of modified nucleotides in the peptidyl transferase center

The importance of modified nucleotides in rRNA is suggested by models of the *E. coli* ribosome in which the majority of the approximately 24 modified nucleotides are clustered around the mRNA-tRNA-peptide complex in the functional center of the ribosome. Three of the modifications in the 23S rRNA of *E. coli* are 2′-*O*-ribose methylations at highly conserved nucle-

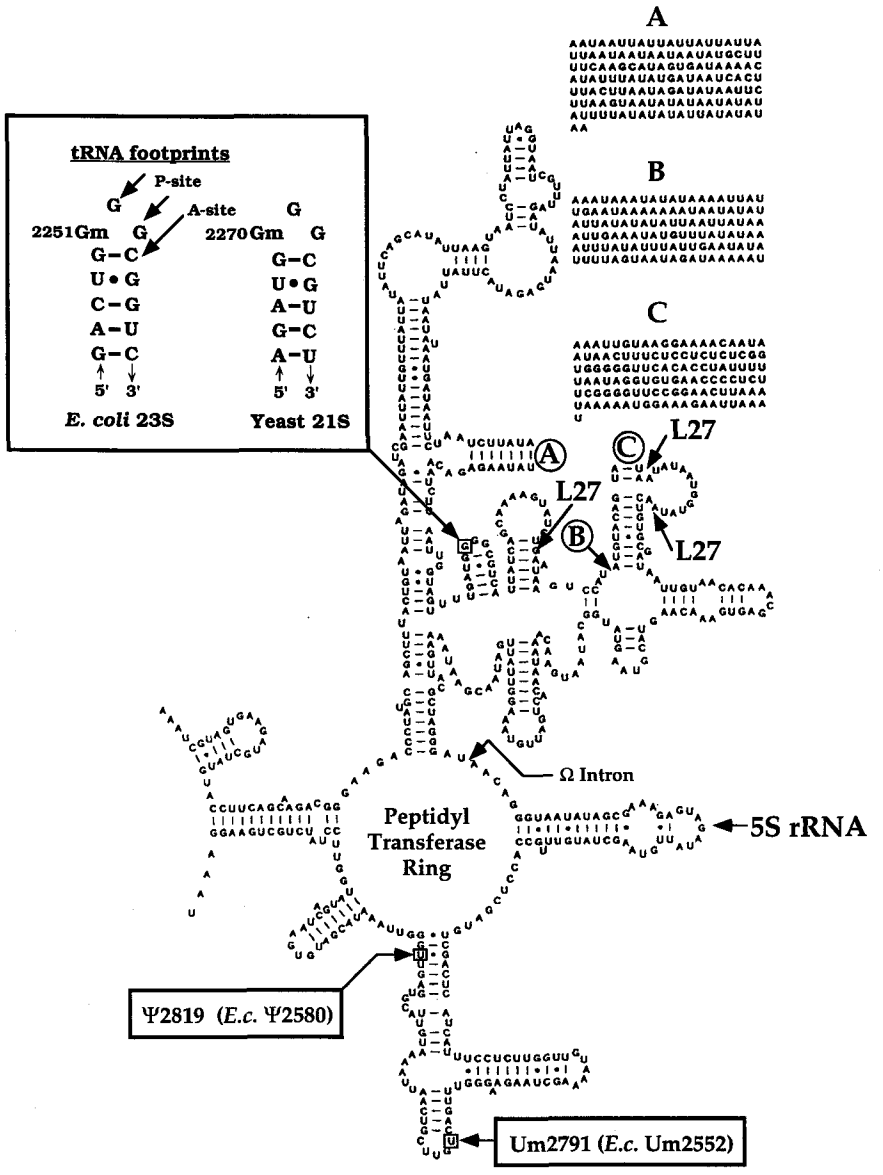

Figure 1. Secondary structure of Domain V in the yeast mitochondrial 21S rRNA. The secondary structure of Domain V of the yeast mitochondrial 21S rRNA [66] is shown with the positions of the three modified nucleotides indicated (see text for refs.). The tRNA footprints in the Gm2251 region of the *E. coli* 23S rRNA are shown in the inset [41]. Arrows indicate positions corresponding to sites where crosslinks to L27 [21, 22] and to 5S rRNA [34, 35] have been detected in Domain V of *E. coli* 23S rRNA. A, B, and C indicate the location of variable expansion segments present in the 21S rRNA, and the location is shown for the optional *Ω* intron in the pre 21S rRNA.

otides in the PTC of Domain V. Yeast mitochondrial 21S rRNA lacks base methylations but retains two ribose methylations [37, 38] and one pseudouridine (Ψ) [39], corresponding to Gm2251, Um2552 and Ψ2580, respectively, in the *E. coli* LSU rRNA (fig. 1). The presence of only three modified nucleotides in the otherwise minimally modified mitochondrial 21S rRNA points to a fundamental role for these particular modifications in ribosome assembly or function or both. Indeed, we have shown that the *PET56* nuclear gene encodes an rRNA ribose methyltransferase (Pet56p) required for the formation of 2′-*O*-methylguanosine at G2270 21S rRNA (G2251 in 23S rRNA) and that purified Pet56p catalyzes the site-specific formation of 2′-*O*-methylguanosine (Gm) on in vitro transcripts of both mitochondrial 21S rRNA and *E. coli* 23S rRNA [40]. Furthermore, *PET56* is essential for the formation of functional mitochondrial ribosomes.

The identification of a specific nucleotide modification that is functionally required in rRNA has important implications for understanding the role of the rRNA in the catalysis of peptide bond formation. Gm2251 in *E. coli* 23S rRNA occupies a prominent position in the PTC; the adjacent bases, G2252 and G2253 are protected from chemical probing by tRNA bound in the P site, and C2254 is protected by tRNA in the A site (see

fig. 1) [41]. There is important recent evidence that binding of the CCA 3′ end of tRNA to the P site requires Watson-Crick pairing between C74 of tRNA and G2552 of 23S rRNA [42]. Ribose methylation at G2251 could influence this interaction. Furthermore, while functional small ribosomal subunits have been reconstituted using unmodified in vitro transcripts of 16S rRNA [43], there are no reports of reconstitution of particles with PT activity using in vitro transcripts of 23S rRNA. Thus, one or more posttranscriptionally modified nucleotides, possibly Gm2251, appear to be required for the assembly of the large subunit.

Is the Pet56p-catalyzed formation of Gm2270 in yeast mitochondrial 21S rRNA required for large subunit assembly or function of the PTC or both? An involvement in assembly is suggested by the analysis of conditional *pet56* mutants. The *his3-Δ200* mutation removes part of the *PET56* promoter region and decreases *PET56* expression by 90%, resulting in a cold-sensitive respiratory growth phenotype and decreased accumulation of the mitochondrial large ribosomal subunit [37, 40]. Additional mutant alleles have been generated by four-codon insertions in the coding region of *PET56*, and one such allele, *pet56-6*, also causes cold-sensitive respiratory growth, deficiency of mature large subunits, and accumulation of slow sedimenting particles containing large subunit r-proteins [37]. These data suggest that *PET56* is required for the assembly of stable large subunit particles. Unfortunately, in the absence of assembly, it is not possible to study the effect of methylation on ribosome function.

While *PET56* is normally essential for the formation of functional mitochondrial ribosomes, an extragenic mutation has been isolated (*SRM1-1*, *s*uppressor of *r*ibose *m*ethylase) that suppresses, albeit very weakly, *pet56* loss-of-function mutations (K. Sirum-Connolly and T. L. Mason, unpublished observation). The *SRM1-1*, *pet56-Δ1* strain lacks detectable methylation at G2270, so the suppressor is not a by-pass mutation. We have not yet been able to clone the suppressor gene; however, further genetic analysis has revealed a genetic interaction between the suppressor allele and the gene for the mitochondrial homologue of *E. coli* L1. Since *E. coli* L1 binds to 23 S rRNA in the peptidyl transferase region of Domain V, less than 60 nucleotides upstream of Gm2251, is involved in tRNA binding to the P-site, and crosslinks to L33, it seemed likely that a mutant form of mitochondrial L33 (MRPL39) might be responsible for the suppression activity. However, the nucleotide sequence of *MRPL39* and its flanking DNA in the *SRM1-1*, mutant is identical to the published sequence of wild-type *MRPL39* (K. Sirum-Connolly and T. L. Mason, unpublished observation). While the analysis of *SRM1-1* suppressor is not complete, the results nevertheless are sufficient to conclude that neither Gm2270 nor Pet56p itself is absolutely essential for the formation and function of the PTC.

Synthesis of *E. coli* large subunit rRNA with only three modified nucleotides

The enzymes responsible for Gm2251, Um2552 and Ψ2580 in 23S rRNA have not been identified in *E. coli*, so it is not yet possible to test the functional properties of transcripts carrying only these conserved modifications in reconstituted *E. coli* 50S subunits. However, since purified Pet56p catalyzes site-specific Gm formation on in vitro transcripts of *E. coli* 23S rRNA, it is possible that the mitochondrial enzymes responsible for Um and Ψ formation will also correctly modify *E. coli* rRNA. To test this possibility, we have engineered the in vivo expression of *E. coli* 23S rRNA in yeast mitochondria using a T7 RNA polymerase system in which T7 RNA polymerase is expressed from a nuclear plasmid and targeted for mitochondrial import [44]. Plasmid DNA containing the *E. coli* 23S rRNA gene under control of the T7 promoter was transformed into [*rho*^{0}] mitochondria which lack all endogenous transcripts. Analysis of modification in the heterologous rRNA transcripts in the transformed mitochondria confirmed the presence of at least two of the three expected nucleotide modifications (C. Sirum-Connolly and T. L. Mason, unpublished observation). Since the mitochondrial enzymes catalyze in vivo site-specific modification of the *E. coli* 23S rRNA, it should be possible to produce minimally modified 23S rRNA in yeast mitochondria for functional analysis in the well-developed system for reconstitution of *E. coli* ribosomes.

Phylogenetic conservation of rRNA ribose methylation

The G at position 2251 in *E. coli* 23S rRNA is universally conserved and the 2′-*O*-methylation of this nucleotide is believed to be equally widespread [45]. Surprisingly, however, homologues of the Pet56p methyltransferase have not been identified in any other organism except *Saccharomyces kluyveri*, a close relative of *S. cerevisiae* (T. King and T. L. Mason, unpublished observation). Database searches for sequences related to Pet56p reveal several confirmed and putative ribose methyltranferases in bacterial species, but none has been confirmed as a functional homologue of Pet56p. It is also surprising that there is no obvious candidate gene in the yeast genome for the enzyme that catalyzes 2′-*O*-methylation of Um2791 in yeast mitochondrial 21S rRNA. It should be noted that eukaryotic cytoplasmic rRNAs contain a large number of ribose methylations. Yeast cytoplasmic rRNAs, for example, contain about 55 sugar-methylated nucleotides [46]. However, eukaryotic cells apparently catalyze site-specific rRNA sugar methylation by a mechanism different from that in prokaryotes, involving fibrillarin-associated small nucleolar RNAs with rRNA complementarity to select the target sites for methylation [47].

New approaches to the genetic analysis of the unusual Var1 protein

Properties of the *VAR1* gene and its protein product

Mitochondrial genomes encode a variable number of r-proteins ranging from several in plants, to one in certain yeasts and filamentous fungi, and none in mammals. The genes for the nuclear-encoded r-proteins were presumably relocated from the mitochondria to the nucleus during evolution. In yeast, Var1p is the only mitochondrial r-protein synthesized in the organelle and the only major protein product of the mitochondrial genetic system that is not part of an energy transducing complex of the inner membrane. The properties of the *VAR1* gene and its protein product have been covered in a recent review and will be only briefly summarized here [48].

Var1p is not related to any known bacterial r-protein, yet it is an essential, stoichiometric component of the yeast mitochondrial small ribosomal subunit [49, 50]. The protein exhibits strain-dependent size polymorphism, hence the name variant protein 1 or Var1 [51]. This size polymorphism is the result of different combinations of optional coding sequences at four positions within the *VAR1* coding region [52]. Two of the these positions are sites for the insertion of a 46-bp GC cluster, which accounts for almost all of the GC content of the gene when it is present. The other optional sequences consist of expansions and contractions of strings of AAT (Asn) codons at two positions in the coding sequence (fig. 1A) [53]. This sequence heterogeneity has no apparent effect on mitochondrial function.

Considering the large number of mutations that have been isolated in yeast mtDNA and the polymorphic nature of *VAR1*, it is surprising that only two mutations have been characterized that affect *VAR1* function, and neither one is located in the *VAR1* coding region [54, 55]. Both mutations affect steps in the processing of *VAR1* mRNA, which is derived from a polygenic transcript. The h56 mutation is a single-base substitution that blocks normal 5′-end cleavage, and the PZ206 mutation is a 207-bp deletion that blocks normal cleavage at the 3′ end of the mRNA [48]. The scarcity of mitochondrial mutations in the *VAR1* gene and the lack of known nuclear mutations that affect *VAR1* expression are most likely due to the instability of mtDNA in the absence of a functional mitochondrial translation system, causing mutants with impaired *VAR1* function to convert to cytoplasmic petite double mutants.

Functional significance of *VAR1*

The unusual properties of Var1p raise interesting questions about its evolutionary origin and functional role in the mitochondrial ribosome. Butow et al. [56] have pointed out that the high AT content and codon usage in *VAR1* more closely resemble spacer sequences and intronic open reading frames than other mitochondrial protein coding sequences and have postulated that *VAR1* might have evolved recently through the shuffling of segments of mtDNA. However, Var1p is apparently involved in an early step in small subunit assembly [50] and is required for the of incorporation of at least one r-protein of eubacterial descent [57]. It appears, therefore, that *VAR1* might have originated in eubacteria and diverged to the point where its lineage cannot be recognized by sequence comparison.

What is the functional significance of a single r-protein gene in the yeast mitochondrial genome? If the mitochondrial progenitor contained a *VAR1* gene and mitochondrial evolution involved the transfer of r-protein genes to the nucleus, then its continued presence in the mitochondrial genome is either fortuitous or else confers a selective advantage to the cell. It is also possible that mitochondrial synthesis of Var1p is obligatory because it cannot be synthesized in the cytoplasm and imported into mitochondria, or perhaps because in situ synthesis is required to accommodate the ribosome assembly pathway.

One attractive possibility for the function of Var1p is an involvement in the uniquely mitochondrial mode of translational initiation that requires the interaction between the small ribosomal subunit and message-specific translation factors [4]. There is good genetic evidence for the functional interaction of three mitochondrial r-proteins, Mrp1p, Mrp17p and Pet123p, with the mRNA-specific translational activator Pet122p [58–60]. Interestingly, these three r-proteins, like Var1p, do not appear to be related to any eubacterial r-protein, suggesting that they might have evolved to provide functions that are unique to the mitochondrial ribosome. *VAR1* mRNA might itself require mRNA-specific translational activators, and if so, do they have different functional properties compared to the proteins that activate translation of mRNAs for integral membrane proteins?

Relocation of *VAR1* to the nucleus

A promising experimental approach to the genetic analysis of the structure, function, and regulation of Var1p is to engineer its expression from a gene in the nucleus. This would permit facile manipulation of the nuclear gene and also allow genetic screens for mutants with defective expression of mitochondrial *VAR1* while maintaining mitochondrial translation and the integrity of [*rho*$^+$] mtDNA with imported Var1p. To test the feasibility of this approach, a universal codon equivalent of *VAR1*, *VAR1*^{u} was created by gene synthesis, fused in-frame to the sequence for the *COX4* leader peptide, and placed under the transcriptional control of the *ADH1* promoter. A construct for expression of

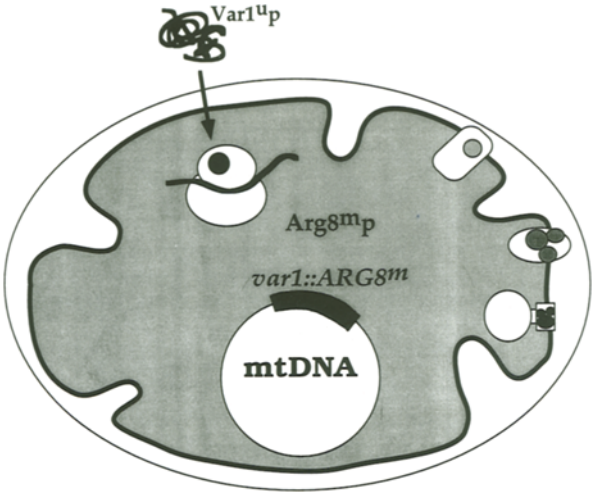

Figure 2. Disruption of the mitochondrial *VAR1* gene by insertion of recoded *ARG8*. Strains with *var1::ARG8^{m}* gene inserted into mitochondrial DNA in place of *VAR1* are dependent on the expression of nuclear *VAR1^{u}* for respiratory growth and arginine prototrophy.

epitope-tagged Var1^{u}p was made by fusing the sequence for influenza HA antigen to the 3′ end of the *VAR1^{u}*. With this system, it was possible to show that cytoplasmically translated Var1^{u}p is efficiently targeted to the mitochondrial matrix where it is incorporated specifically into the small ribosomal subunit [48] (M. E. Sanchirico and T. L. Mason, unpublished observation). Ribosomes containing Var1^{u}p are functional as shown by the ability of *VAR1^{u}* to complement the PZ206 *VAR1* mutation in mitochondrial DNA [48]. However, since the PZ206 mutant has a leaky phenotype and readily reverts due to a nuclear suppressor mutations [61], complementation of PZ206 does not provide conclusive evidence that *VAR1^{u}* is able to completely replace the function of mitochondrial *VAR1*.

Disruption of the mitochondrial *VAR1* gene

A stable *VAR1* knockout mutant with an absolute requirement for *VAR1^{u}* was generated by replacing the *VAR1* coding sequence with a recoded nuclear gene (*ARG8*) for the mitochondria-localized arginine biosynthetic enzyme acetylornithine aminotransferase [62] (M. E. Sanchirico and T. L. Mason, unpublished observation). Strains with this *var1::ARG8^{m}* gene inserted into mitochondrial DNA in place of *VAR1* are dependent on the expression of nuclear *VAR1^{u}* for respiratory growth and arginine prototrophy (fig. 2) (M. E. Sanchirico and T. L. Mason, unpublished observation). Transcription of *VAR1^{u}* from the *MRP7* promoter supports normal respiratory growth, indicating that Var1^{u}p is fully functional at an expression level similar to a nuclear encoded mitochondrial r-protein. Although Var1^{u}p maintains mitochondrial protein synthesis in the absence of *VAR1*, thereby preserving the integrity of mitochondrial DNA, it does not prevent conversion to [*rho*$^{-}$] in strains lacking another essential r-protein. Therefore, Var1p is apparently not the mitochondrial translation product required for the integrity of the mitochondrial genome.

The successful relocation of *VAR1* function from the mitochondrion to the nucleus rules out an intrinsic barrier to the expression of Var1p from a nuclear gene. *VAR1* is therefore another example of a gene for which there is no compelling reason for its persistence in mitochondrial DNA. Only two proteins, cytochrome *c* oxidase subunit I and apocytochrome *b*, are exclusively encoded by the mitochondrial genome and are therefore candidates for obligatory synthesis in mitochondria [63]. Significantly, experiments in yeast with a recoded *COB* gene support the idea that a large number of hydrophobic transmembrane helices in a polypeptide presents a significant barrier to import into mitochondria [64]. Perhaps the entire mitochondrial genetic system is retained only for the synthesis of two hydrophobic proteins and the presence of genes for proteins such as Var1p is simply fortuitous.

Aside from evolutionary implication, the functional relocation of *VAR1* will greatly facilitate the molecular genetic analysis of mitochondrial ribosomes, and Var1p in particular. By moving *VAR1* to the nucleus, the formation of the mitochondrial ribosome has been made independent of their function. This provides an excellent experimental system for the in vivo analysis of mutations in nuclear genes for mitochondrial r-proteins, including *VAR1^{u}*, as well as mutations introduced into rRNA genes in mitochondrial DNA. Moreover, it will now be possible to screen for mutants with defective function of mitochondrial *VAR1* while maintaining an active mitochondrial translation system with *VAR1^{u}*. The *var1::ARG8^{m}* gene can be used as a reporter gene to study *VAR1* regulation and to identify mutations that affect the expression of *VAR1*.

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