Identification of the yeast nuclear gene for the mitochondrial homologue of bacterial ribosomal protein L16

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ABSTRACT
An open reading frame encoding a member of the L16 family of ribosomal proteins is adjacent to the URA7 gene on the left arm of chromosome II in Saccharomyces cerevisiae. The predicted L16-like polypeptide is basic (pi 11.12), contains 232 amino acids (26.52 kDa) and has 36% amino acid sequence identity to E.coli L16. Immunoblot analysis with polyclonal antibodies to the L16-like polypeptide showed specific cross-reaction with a 22 000 Mr mitochondrial polypeptide that co-sediments with the large subunit of the mitochondrial ribosome in sucrose density gradients. The levels of the L16 mRNA and protein varied in response to carbon source. In [rho°] cells lacking mitochondrial rRNA, the L16 mRNA accumulated at normal levels, but the protein was barely detectable, indicating RNA-dependent accumulation of the L16 protein. Gene disruption experiments demonstrated that the yeast mitochondrial L16 is an essential ribosomal protein in vivo.

INTRODUCTION
The mitochondrial ribosome is composed of both nuclear and mitochondrial encoded components. In Saccharomyces cerevisiae, the large (21S) and small (15S) rRNAs and the var1 protein in the small ribosomal subunit are encoded by the mitochondrial genome, whereas the remaining ribosomal proteins (r-proteins) are specified by nuclear genes, synthesized on cytoplasmic ribosomes and imported by the mitochondria (for reviews, see refs 1–4). Compared with the Escherichia coli ribosome, the yeast mitochondrial ribosome is relatively protein rich with as many as 48 proteins in the 54S large subunit and 34 proteins in the 37S small subunit (4).

The nuclear genes for over one-third of the mitochondrial r-proteins have been identified and cloned, but, interestingly, only about one-half of the predicted protein sequences show significant relatedness to other r-proteins. We are interested in the structure of the peptidyl transferase center in the yeast mitochondrial ribosome and mitochondrial homologues of proteins implicated in the peptidyl transferase center of the well-studied E.coli ribosome provide extremely valuable subjects for comparative analysis. To date, three such proteins have been identified, including the mitochondrial homologues of L2 (Pan and Mason, submitted), L3 (5) and L27 (6).

We previously reported the presence of an open reading frame capable of encoding a mitochondrial homologue of bacterial r-protein L16 (7). In this report, we confirm that the polypeptide product of this open reading frame is a component of the mitochondrial large ribosomal subunit. This finding is particularly significant because there is abundant evidence from in vitro studies that E.coli L16 is located in the peptidyl transferase center (8,9) and is an important participant in the reconstitution of peptidyl transferase activity (10,11). Under certain conditions, however, reconstituted ribosomal particles lacking L16 are active in poly(Phe) synthesis (12), suggesting that L16 is mainly a structural component and is not directly involved in the catalysis of peptide bond formation. L16 has not been subjected to mutational analysis in E.coli, so it is not yet clear whether L16 is an essential r-protein in vivo. Here we confirm the identity of the nuclear gene for the yeast mitochondrial L16 protein, provide information about its expression and show that mitochondrial L16 is an essential r-protein in vivo.

MATERIALS AND METHODS
Isolation of an RML16 clone and plasmid construction
A DNA fragment containing RML16 and its 5' and 3' flanking sequences was cloned from a library of 4–5 kb EcoRI fragments of genomic DNA (strain 22-2D) ligated into the EcoRI site of Bluescript KS+. The RML16-containing plasmid pCP201 was identified by colony hybridization with a 408 bp sequence from the RML16 coding region. This hybridization probe was generated by PCR amplification using the primer sequences GGATGGTGAGGGTACCCACAGGG from within the RML16 coding region and GTGTTTCTTCAGCGCTCATCCGGG from the 3' flanking sequence, which were designed according to published sequence information (13). pCP205 was obtained by insertion of a BamHI linker (CGGGATCCCG) at the Clal site in the 3' flanking sequence of RML16 in pCP201. pCP209 contains an rml16Δ::URA3 allele constructed by subsequent removal of

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the 550 bp BamHI fragment of pCP205 encoding the C-terminal 167-amino-acids of Rm16p and its replacement with the 3.8 kb BamHI–BglII fragment of pNKY51 (14), which contains the URA3 gene flanked by the hisG repeats. For T7-promoter driven expression of Rm16p sequences in E.coli, the 550 bp BamHI fragment of pCP205 was inserted at the BamHI site of pET-3a (15), generating pCP211.

Saccharomyces cerevisiae strains and media

The following yeast strains were used: 22-2D (MATα ura3-52 trp1–Δ101 leu2-3,112 cyh2 can1 [rho+]), provided by G. R. Fink; the isonuclear [rho+], [F1L, rho−], and [rho−] derivatives of Cop161-U7 (MATα ade2-101 lys2-801 ura3-52), provided by R. A. Butow. The diploid strain CPY251 (MATα/α ade2-101/+ trp1–Δ101/+ ura3-52/ura3-52 leu2-3,112/+ cyh2/+ [rho+]) resulted from mating 22-2D [rho+]/[rho+] to Cop161-U7 [rho+]. Yeast transformations were performed using the simplified lithium acetate procedure described by Elble (16). CPY252 (MATα/α ade2-101/+ trp1–Δ101/+ ura3-52/ura3-52 leu2-3,112/+ cyh2/+ rml16Δ::URA3/RML16 [rho+]) was generated by transforming CPY251 with the rml16Δ::URA3 allele contained on the 6.1 kb Clal–EcoRI fragment from pCP209. CPY252-1A, CPY252-1B, CPY252-1C and CPY252-1D are spores from a tetrad obtained by sporulation of CPY252; spores CPY252-1B and CPY252-1D carry the rml16Δ::URA3 allele.

Purification of recombinant Rm16p and production of polyclonal antisera

A 167-amino-acid C-terminal fragment of Rm16p was overexpressed from the E.coli BL21(DE3), pLysS. The 19 kDa protein accumulated at very high levels in inclusion bodies. The recombinant Rm16 polypeptide was purified from the inclusion bodies by preparative SDS–polyacrylamide gel electrophoresis followed by electro-elution and was used to generate polyclonal antisera in mice as described earlier (17).

Analysis of nucleic acids and proteins

Total yeast cellular DNA and RNA were extracted from yeast cells as previously described (18). RNA was fractionated by electrophoresis in 1.1% agarose–formaldehyde gels (19). Southern and Northern blot analysis and colony hybridization were performed using GeneScreen Plus membranes and Colony/Plaque Screen™ filters from DuPont according to the manufacturer’s instructions. The hybridization probes were 32P-labeled as described (20). Immunoblot analysis of ribosomal proteins in yeast subcellular fractions and in fractions from sucrose density gradients were performed as described previously (18).

RESULTS

An open reading frame for an L16-like protein was found in the flanking DNA of the published sequence of the S.cerevisiae URA7 gene (13,21). This open reading frame is capable of encoding a basic protein of 232 amino acid with a calculated molecular mass of 26.52 kDa and a pl of 11.12. The codon usage in this open reading is similar to yeast proteins expressed at low levels, including several mitochondrial ribosomal proteins. In addition, the yeast L16-like protein has 36% amino acid sequence identity to E.coli L16. Taken together, these data suggest that the open reading frame specifies the yeast mitochondrial homologue of E.coli L16 and it was named RML16 for ribosomal mitochondrial protein L16 (this designation follows the nomenclature for yeast mitochondrial r-proteins suggested by B. Baum, pers. comm.). A genomic clone containing RML16 was obtained on a 4.5 kb EcoRI fragment from the yeast strain 22-2D. The restriction map of the rml16Δ::URA3 allele was constructed by replacing the 550 bp BamHI fragment of the RML16 gene with the 3.8 kb BamHI–BglII URA3 fragment. The 6.1 kb Clal–EcoRI fragment containing the rml16Δ::URA3 allele was used to transform the wild-type diploid Ura+ strain CPY251 to obtain the Ura+ strain CPY252. (B) Southern blot analysis. Total DNA from each of the four spores of a tetrad from the RML16::URA3 diploid strain CPY252 was digested with EcoRI and subjected to Southern blot analysis using the 32P-labeled 1.88 kb Clal fragment of pCP201 as the hybridization probe (see A). (C) Immunoblot analysis. Mitochondrial proteins (75 μg per lane) from the four spores shown in B were separated by SDS–PAGE (12.5%) and subjected to immunoblot analysis with the mouse polyclonal antibodies to Rm16p and [125I]-goat anti-mouse Ig secondary antibody. The autoradiograph shows the immunoreactive 22 kDa Rm16p.
respiration in the heterozygous diploids. From these crosses were uniformly Per, indicating that the RML16 \( \rho^0 \) haploid tester strains devoid of mitochondrial DNA synthesis is required to maintain the integrity of + mitochondrial RNA. Since it is well documented that yeast mitochondrial protein \( \rho^0 \) (either 22-2D or Copl61-U7, \( \rho^0 \)).

Randomly isolated subclones of several rml16A::URA3 spores to gene-disruption mutants was examined genetically by crossing RML16 spores (spores B and D) and, as shown + replaced prototrophy and respiratory growth on nonfermentable carbon sources. All of the tetrads contained two Ura+ diploid strain CPY251. RML16 was used to replace, by homologous recombination, one of the mitochondrial DNA (6,22), these results indicate that RML16 encodes an essential component of the mitochondrial translational apparatus.

The regulation of RML16 in response to carbon source and mitochondrial genotype was examined by Northern and immunoblot analysis as shown in Figure 3. The levels of the RML16 transcript and protein are both reduced in cells growing fermentatively on glucose as compared to cells growing on the nonfermentable carbon sources glycerol plus 2% ethanol (GE). The regulation of RML16 and Rm16p levels in response to catabolite repression. The isogenic \( \rho^+ \), [F11, \( \rho^+ \)] and \( \rho^0 \) derivatives of strain Copl61-U7 were grown in rich media with one of the following carbon sources: 5% glucose (Glu), 2% galactose (Gal) or 2% glycerol plus 2% ethanol (GE). The isogenic \( \rho^+ \), [F11, \( \rho^+ \)] and \( \rho^0 \) cells, which express respectively, both the small and large mitochondrial r-proteins (6,18). The levels of RML16 transcripts were not differentially affected in \( \rho^0 \) or \( \rho^0 \) derivatives of strain [Wur,F11, \( \rho^0 \)].

Figure 2. Immunoblot analysis of Rml16p in mitochondrial ribosomal subunits separated by sucrose gradient centrifugation. Mitochondrial ribosomal subunits from 22-2D cells grown in YPGE media at 30°C were resolved by sucrose gradient centrifugation in the presence of 500 mM NH4Cl as described previously (18). Rml16p was detected in the gradient fractions by immunoblot analysis using polyclonal antibodies to Rml16p. The A260 profile of the gradient is shown at the top and the autoradiograph of the immunoblot is aligned under the profile. The positions of the small and large subunits are indicated by arrows. The lane marked 'mt' contains total mitochondrial protein (50 \( \mu \)g).

Figure 2 indicates that Rml16p is located in the mitochondrial fraction (lane labeled 'mt') and co-sediments specifically with the 54S large ribosomal subunit.

To test whether Rml16p is an essential protein in the mitochondrial ribosome, an rml16\( \Delta \)::URA3 gene disruption allele (Fig. 1A) was used to replace, by homologous recombination, one of the mitochondrial copies of RML16 in the Ura- diploid strain CPY251. The Ura+ rml16\( \Delta \)::URA3/RML16 diploid CPY252 was sporulated and 24 tetrads were examined for the segregation of Ura+ prototrophy and respiratory growth on nonfermentable carbon sources. All of the tetrads contained two Ura+, respiration-deficient (Per-) spores. Southern blot analysis of a representative tetrad (Fig. 1B) confirmed that the rml16\( \Delta \)::URA3 disrupted gene had replaced RML16 in the Ura+ spores (spores B and D) and, as shown in the immunoblot in Figure 1C, these spores also lacked the Rml16 protein. The stability of the mitochondrial DNA in the gene-disruption mutants was examined genetically by crossing randomly isolated subclones of several rml16\( \Delta \)::URA3 spores to RML16 [rho+] haploid tester strains devoid of mitochondrial DNA (either 22-2D [rho+] or Copl61-U7, [rho+]). The diploids issued from these crosses were uniformly Per+, indicating that the rml16\( \Delta \)::URA3 mutants had quantitatively converted to [rho+] or [rho+] cytoplasmic petites and therefore were unable to restore respiration in the RML16/ rml16\( \Delta \)::URA3 heterozygous diploids.

Since it is well documented that yeast mitochondrial protein synthesis is required to maintain the integrity of [rho+] mitochondrial DNA (6,22), these results indicate that RML16 encodes an essential component of the mitochondrial translational apparatus.

DISCUSSION

In this report, we have confirmed that an open reading frame closely linked to URA7 on chromosome II in the genome of S.cerevisiae encodes the mitochondrial equivalent of bacterial ribosomal protein L16. The bacterial L16 protein has been extensively studied in vitro. Reconstitution studies show that L16 is a late assembly protein (12) and immunoelectron microscopy places it on or near the subunit interface, in the region identified as the peptidyl transferase center, adjacent to the central protuberance of the 50S particle (23). The results of several additional lines of
The alignment in Figure 4 includes three L16 proteins that are r-proteins, is somewhat larger than the equivalent protein in E.coli.

RML16 NPYDAKKPP SXYNLIKS QEPQKLFGR 183

Figure 4. Sequence alignment of Rml16p with members of the L16 protein family. The alignment of Rml16p with eight L16 sequences selected from the 22 sequences currently available in L16 protein family was derived using the PileUp program of the GCG Sequence Analysis Software Package, Version 7.3 (34). Identical residues between Rml16p and the rest of the family members are shaded. The Rml16p sequence was started from the first AUG codon and the amino acids were numbered according to Rml16p. EcoL16, E.coli L16 (35,36); BsuL16, Bacillus subtilis L16 (37); Mpo-mito, Marchantia polymorpha mitochondrial L16 (38); maize-mito, Zea mays mitochondrial L16 (39); Pethy-mito, Petunia hybrida mitochondrial L16 (40); Cpa-chl, Cyanophora paradoxa chloroplast L16 (41); Cre-chl, Chlamydomonas reinhardtii chloroplast L16 (42).

experimentation, including single omission reconstitution (10–12), protein-tRNA cross-linking (8,9) and antibiotic binding (24,25), indicate that L16 plays an important role in the assembly and structure of the peptidyl transferase center, but it does not seem to be essential for translation, per se.

Information is lacking about the functional requirement for L16 in vivo, although the failure to isolate L16 mutants in E.coli has been cited as indirect evidence of the essential nature of L16 (12,26). It is significant, therefore, that disruption of RML16 provides the first direct evidence that an L16 protein is an essential ribosomal component in vivo, at least in yeast mitochondria. However, two examples emphasize the need for caution in extrapolating from one ribosomal system to another. The yeast mitochondrial counterparts of bacterial L27 (6,7) and L30 (27) are essential, as judged by the instability of mitochondrial DNA in mutants lacking either of these proteins, whereas E.coli mutants lacking L27 or L30 are viable (26,28). Additional studies are needed to determine whether L16 is an essential component of the bacterial ribosome in vivo.

Figure 4 shows the predicted Rml16p sequence aligned with eight representatives selected from the over 20 known members of the L16 protein family. In comparison to other L16 proteins, Rml16p has an extension of ~40 to 50 amino acids at the C-terminus. Thus, Rml16p, like several other yeast mitochondrial r-proteins, is somewhat larger than the equivalent protein in E.coli. The alignment in Figure 4 includes three L16 proteins that are predicted from gene sequences in the mitochondrial genomes of plants. L16 coding sequences are also found in the mitochondrial genomes of the chlorophyte alga Protophcea wickerhamii (29) and the amoeboid protozoan Acanthamoeba castellanii (30). Seven of the eight identified genes for mitochondrial L16 proteins are located in mitochondrial DNA; RML16 is the only one located in the nuclear genome.

The alignment of the predicted L16 proteins shows that Rml16p and the protein encoded by the maize mitochondrial genome have 41-amino-acid N-terminal extensions (Fig. 4). While this extension sequence might contain the mitochondrial targeting information for the yeast mitochondrial protein, there is no apparent need for such a targeting sequence in a polypeptide translated within maize mitochondria. Indeed, it has been argued that translation of L16 in the plant mitochondria initiates at a GUG valine codon instead of at an upstream AUG, then these proteins would also lack N-terminal extensions and their N-termini would align with that of E.coli L16. Since there are three in-frame AUG codons near the 5' end of the RML16 open reading frame, there is also uncertainty about the nature of the N-terminal extension in Rml16p. Further analysis will be required to determine which
AUG codon is used to initiate Rml16p translation and whether the protein is synthesized with a cleavable mitochondrial targeting presequence.

Our results show that the yeast mitochondrial L16 protein is expressed from the \textit{RML16} nuclear gene, but the evidence is less clear for the functional expression of the L16 genes in mitochondrial genomes. For example, a premature stop codon created by RNA editing is thought to make the \textit{Petunia} mitochondrial \textit{rpl16} gene nonfunctional (33). Therefore, some organisms might require information about the sequence of the \textit{RML16} gene and to Dr Ozier-Kalogeropoulos for providing unpublished \textit{S.cerevisiae} URA7 reading frame in the flanking sequence of the \textit{RML16}\textit{-}\textit{a} functional \textit{rpl16} RNA editing is thought to make the mitochondrial \textit{Petunia} mitochondrial \textit{rpl16} RNA editing is thought to make the mitochondrial \textit{Petunia} mitochondrial \textit{rpl16} RNA editing is thought to make the mitochondrial

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\section*{REFERENCES}