Structural and functional analyses of a yeast mitochondrial ribosomal protein homologous to ribosomal protein S15 of *Escherichia coli*

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ABSTRACT

We have purified a small subunit mitochondrial ribosomal protein, MRPS28p, from the yeast, *Saccharomyces cerevisiae*. Sequence from the amino terminus of MRPS28p was used to design a degenerate oligonucleotide that was complementary to the MRPS28 gene. The MRPS28 gene was isolated and its sequence determined. The *MRPS28* sequence encodes a 28 kDa protein that has a region of homology with ribosomal protein S15 of *E. coli*. This region spans the entire length of the *E. coli* protein, but as MRPS28p is larger, includes only the portion of the MRPS28p sequence from amino acids 150 to 238. Based on this homology, we predict that MRPS28p, like *E. coli* S15, interacts directly with small subunit rRNA and functions as an early protein in ribosome assembly. Cells carrying a disrupted chromosomal copy of *MRPS28* are unable to respire and spontaneously lose portions of their mitochondrial genomes at a high frequency. These phenotypes are consistent with an essential role for MRPS28p in the assembly and/or function of the mitochondrial ribosome.

INTRODUCTION

The mitochondrial ribosome is one of several complexes within this organelle containing components coded by the nuclear and mitochondrial genomes (1,2). In the yeast, *Saccharomyces cerevisiae*, the two rRNAs and a single protein of the small ribosomal subunit are coded by the mitochondrial genome. The remaining 60−70 ribosomal proteins are coded in the nucleus, translated in the cytoplasm, and must be imported into the mitochondrial matrix where subunit assembly occurs. Nine nuclear genes have been isolated from yeast that are reported to code for mitochondrial ribosomal proteins: *MRP1* (3), *MRP2* (3), *MRP7* (4), *MRP13* (5), *MRP-L8* (6), *MRP-L20* (6), *YML-31* (7), *YMR-31* (8), and *YMR-44* (8). Of these genes, three show sequence similarity with genes coding for ribosomal proteins from *E. coli*. These genes as well as the mitochondrially-encoded rRNAs have likely descended from a eubacterial endosymbiotic ancestor of mitochondria (9,10). The mitochondrial ribosomal proteins without recognizable homologs in *E. coli* have either diverged far enough away from their eubacterial ancestors to preclude meaningful sequence comparisons or they may have

been recruited from elsewhere in the eukaryotic cell to function in mitochondrial protein synthesis (4).

To better define the structure/function relationships between eubacterial and mitochondrial ribosomal proteins, it is necessary to identify mitochondrial proteins homologous to eubacterial proteins for which clearly defined roles in ribosome assembly and/or function have been established. Functional assignments for ribosomal proteins remain rather vague because no single function in protein synthesis has been attributed solely to an individual ribosomal protein (11). However, certain ribosomal proteins have been shown to play specific, well-defined roles in ribosome assembly (11,12). Best characterized in this regard are the proteins that interact directly with rRNA and function early in the ribosome assembly pathway (13). If mitochondrial homologs of the early assembly proteins of *E. coli* can be found, specific questions related to the structure and function of this group of ribosomal proteins can be addressed.

We report here the purification of MRPS28p, a small subunit mitochondrial ribosomal protein from *Saccharomyces cerevisiae*, and the isolation of its gene. A portion of the MRPS28p sequence, deduced from its gene, has homology with ribosomal protein S15 of *E. coli*. *E. coli* S15 (Ec S15) has been extensively characterized and is known to interact directly with 16S rRNA and function at early steps in ribosome assembly (12). The region of 16S rRNA that interacts with Ec S15 has been mapped (14−16) and is part of a core structure common to virtually all known 16S rRNA-like molecules including yeast mitochondrial 15S rRNA (17). Thus, the sequence conservation between MRPS28p and Ec S15 is likely a consequence of constraints on sequence divergence imposed by the need for these two proteins to functionally interact with conserved regions of small subunit rRNAs. Consistent with an essential role for MRPS28p in the assembly and/or function of the mitochondrial ribosome is that cells disrupted in the chromosomal copy of *MRPS28* display phenotypes characteristic of cells with defects in mitochondrial protein synthesis.

MATERIALS AND METHODS

Yeast and bacterial strains

The yeast strains used in these studies are W303-1B (α ϱ^+ *ade2-1 his3-11 leu2-3,112 ura3-1 trp1-1 can1-100*), and COP161 (*a* ϱ° *ade lys*). The *E. coli* strain used was JM101.

Preparation of yeast mitochondrial ribosomal subunits

Yeast were grown to late logarithmic phase in media containing 1% Bacto-yeast extract, 2% Bacto-peptone, 2% galactose, and 0.2% glucose. Spheroplasts were prepared using yeast lytic enzyme, 70,000 units/g (ICN Biomedicals, Inc.). Spheroplasts were disrupted by rapid decompression using a PARR bomb (18) and mitochondria were prepared from these extracts as described by Chen and Martin (19) except that the final digitonin step was omitted. Mitochondria were purified further by flotation gradient centrifugation to minimize contamination with cytoplasmic ribosomes (20). Mitochondria were lysed with 1% deoxycholate and the lysate was clarified by centrifugation at 14,000×g for 10 minutes. Ribosomes were isolated by centrifugation at 180,000×g for 14−16 hours through a 1M sucrose cushion containing 500 mM NH_4Cl (21). Ribosomal subunits were separated by centrifugation at 85,000×g for 18 hours through 10−30% sucrose gradients under dissociating conditions (22). Gradients were fractionated and the absorbance at A_{254} was monitored using an ISCO type 185 density gradient fractionator and a UA-5 absorbance detector, respectively. Fractions containing the small mitochondrial ribosomal subunits were pooled and the subunits collected by centrifugation at 100,000×g for 16 hours. Mitochondrial ribosomal subunits prepared in this manner were shown to be free of contaminating cytoplasmic ribosomes by immunoblotting with antisera raised to the cytoplasmic ribosomal protein L16 (data not shown). The antisera raised against L16 was kindly provided by Dr. John L. Woolford, Carnegie Mellon University.

Purification of mitochondrial ribosomal proteins by reverse phase HPLC

Proteins were extracted from mitochondrial ribosomal subunits with 66% acetic acid (23). Ribosomal proteins were dialyzed against 20% acetic acid for 2 hours followed by extensive dialysis against 4% acetic acid. Dialysis was carried out at 4°C. Ribosomal proteins were purified by reverse phase HPLC using methods developed for *E. coli* ribosomal proteins (24,25) with the elution conditions adjusted to enhance the separation of mitochondrial ribosomal proteins. Briefly, ribosomal proteins were separated using a 25 mm×4.6 mm Vydac C-18 column, 300 Å pore size. Typically, 200−500 μg of total subunit protein was loaded onto a column for preparative separations. Proteins were eluted from the C-18 column with a gradient of acetonitrile in 0.1% triflouroacetic acid. Elution was carried out at a flow rate of 0.5 ml/minute over a period of 4 hours at room temperature. A detailed description of the gradient used to separate mitochondrial ribosomal proteins is provided in Fig. 1. The eluate was monitored at 214 nm and fractions corresponding to absorbance peaks were pooled, lyophilized, and a portion of each sample was subjected to SDS-polyacrylamide gel electrophoresis (26). The size-fractionated proteins were silver stained and peaks composed of a single protein of greater than 85% purity were subjected to gas-phase sequencing and analysis using an Applied Biosystems 470A Sequencer equipped with a 120A PTH Analyzer. Sequence derived from the amino terminus of one of these proteins, MRPS28p, was used to derive a degenerate oligonucleotide by reverse translation for subsequent use in the isolation of its gene.

DNA manipulations

Yeast genomic DNA was prepared by a modification of the method of Davis *et al.* (27) except that diethyl oxidiformate

treatment was omitted and phenol/chloroform extraction followed by ether extraction and ethanol precipitation was added after the RNase treatment. Genomic DNA was digested with a variety of restriction enzymes and DNA fragments were size-fractionated by electrophoresis through agarose gels. After electrophoresis, DNA fragments were transferred to nitrocellulose according to Southern (28). The MRPS28 oligonucleotide mixture was labelled at its 5' end by T4 polynucleotide kinase and γ-[^{32}P]-ATP. The labelled oligonucleotide mixture was hybridized with yeast genomic DNA at several temperatures. At 60°C only a single fragment of yeast genomic DNA hybridized to the MRPS28 oligonucleotide mixture (Fig. 3). BglII digests of yeast genomic DNA gave a 3.3 kb fragment that hybridized with the MRPS28 oligonucleotide mixture. This fragment was isolated by first size-fractionating preparative digests of genomic DNA by electrophoresis through an agarose gel. DNA fragments in the 3 kb size range were electroeluted from the gel (29) and purified by NACS chromatography (Bethesda Research Laboratories). The genomic DNA fragments were ligated into the unique BamHI site of Bluescript KS (Stratagene), transformed into JM101 cells, and colonies were screened with the MRPS28 oligonucleotide mixture.

Two colonies that hybridized with the MRPS28 oligonucleotide mixture were shown by restriction enzyme mapping to contain the same insert in opposite orientations. Subclones of the 3.3 kb insert suitable for sequencing were generated by unidirectional deletions (30) using an ExoIII/mung bean nuclease deletion kit (Stratagene). DNA was sequenced by the chain termination method of Sanger *et al.* (31) using a Sequenase kit from US Biochemicals. The sequencing strategy is outlined in Fig. 4.

The MRPS28 gene was disrupted by replacing approximately 70% of its coding region with the yeast URA3 gene. One of the deletion subclones used in sequencing that lacked both the HincII and HindIII sites in the polycloning region of the Bluescript vector was used as the starting material to create a disrupted copy of *MRPS28*. An outline of the gene disruption strategy is shown in Fig. 7. The deletion subclone was digested with HincII and HindIII, each of which cleave the plasmid only once within the MRPS28 gene. The HincII-HindIII fragment was separated and the remaining portion of the plasmid was ligated to a HindIII-SmaI fragment containing the yeast URA3 gene and transformed into JM101 cells (29). From the resulting plasmid, a SnaBI-SacI fragment carrying the disrupted copy of *MRPS28* and approximately 300 bases of flanking sequences on both the 5' and 3' ends was isolated and used to transform the yeast strain W303−1B by the alkali metal protocol of Ito *et al.* (32). Transformants were selected by prototrophic growth on uracil-deficient media supplemented with several amino acids and adenine (33). Colonies were replica plated onto YEPG media (1% yeast extract, 3% ethanol, 2% peptone, and 2% glycerol) and respiratory-deficient transformants were screened for a disrupted chromosomal copy of *MRPS28* by Southern analysis (Fig. 3 B).

RESULTS

Purification of proteins of the small subunit of mitochondrial ribosomes and the isolation of a nuclear gene coding for one of these proteins

As part of our ongoing studies on the structure, function, and assembly of mitochondrial ribosomes we have purified several small subunit proteins to near homogeneity by reverse phase HPLC. A typical elution profile of small subunit ribosomal

proteins separated by a Vydac C-18 column with a gradient composed of solution A (0.1% aqueous TFA, 10% acetonitrile) and solution B (0.1% TFA, 55% acetonitrile) is shown in Fig. 1. Peaks were pooled, lyophilized, and the degree of purity of proteins in individual peaks was examined by SDS polyacrylamide gel electrophoresis followed by silver staining (data not shown). Several of the peaks shown in Fig. 1 contained a single protein of greater than 85% purity as judged by silver staining (data not shown). These proteins were used for amino acid sequence analysis.

Fig. 2 shows a partial sequence derived from the amino terminus of a small subunit protein contained within the shaded peak of Fig. 1. This protein will hereafter be referred to as MRPS28p (mitochondrial ribosomal protein, small subunit, 28 kDa). Also shown in Fig. 2 is the sequence of a degenerate oligonucleotide, derived by reverse translation, that was used to isolate the nuclear gene coding for this protein. Yeast genomic DNA was probed with the MRPS28 oligonucleotide mixture over a range of hybridization temperatures. Fig. 3A shows that at 60°C the MRPS28 oligonucleotide mixture hybridized to a single BglII fragment of yeast genomic DNA which was subsequently cloned and sequenced as described in 'Materials and Methods'. The sequencing strategy is outlined in Fig. 4.

Sequence analysis

The nucleotide sequence of the MRPS28 gene is shown in Fig. 5. This region contains an open reading frame capable of coding for a protein of 286 amino acids. The amino acid sequence derived from the amino terminus of the purified MRPS28 protein is identical to the amino acid sequence predicted from the MRPS28 gene from serine 34 to lysine 53. The amino terminus of the purified protein does not coincide with the amino acid

sequence predicted from the beginning of the MRPS28 open reading frame, probably because MRPS28p is initially synthesized as a precursor protein that is proteolytically processed upon entering mitochondria. If the +1 ATG at the beginning of the open reading frame codes for the translation start site, the MRPS28p precursor would have an amino-terminal extension of 33 amino acids. The amino acid composition of the amino-terminal extension is rich in arginine, serine, and leucine and devoid of acidic residues, consistent with a role for these sequences in targeting MRPS28p to the mitochondrial matrix (34). Further analysis of these sequences revealed the MRPS28p precursor may belong to a subset of mitochondrial proteins that are processed in two steps (35).

Comparison of the sequence of MRPS28p with sequences in the GenPept database using the FASTA search program (36) revealed that a portion of MRPS28p had sequence similarity with *E. coli* ribosomal protein S15. Fig. 6 shows that MRPS28p and Ec S15 have 38% sequence identity over 89 amino acids without introducing gaps. This region of similarity covers the entire length of the *E. coli* protein but only the portion of the MRPS28p sequence from residues 150 to 238. Proteins related to Ec S15 have also been identified in other eubacteria (37), chloroplasts (38), and archaebacteria (39). The alignment of four S15-like sequences from *S. cerevisiae* mitochondria, *N. tabacum* chloroplasts, *E. coli*, and the archaebacterium, *H. marismortui* is shown in Fig. 6. A one on one comparison of the
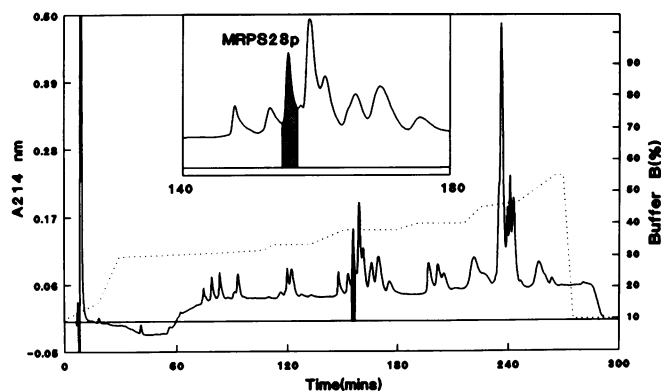
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Ser Ala Lys Ala Val Lys Phe Leu Lys Ala Gln Arg Arg Lys Gln Lys Asn Glu Ala Lys

       3'- TTT CGN GTT GCN GCN TTT GTT TTT TTA CTT CGN TT -5'
           C       C T   T        C   C   C   G   C
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Fig. 2. Amino-terminal sequence of purified MRPS28p and the degenerate oligonucleotide used to isolate its gene. Purified MRPS28p was subjected to successive cycles of Edman degradation and the PTH-amino acids released in each cycle were resolved by reverse phase HPLC and identified by their characteristic retention times. The letter N in the oligonucleotide indicates that all four bases were inserted at this position. Two-fold degeneracy at a position in the oligonucleotide is indicated by placing one base below the other in the sequence.
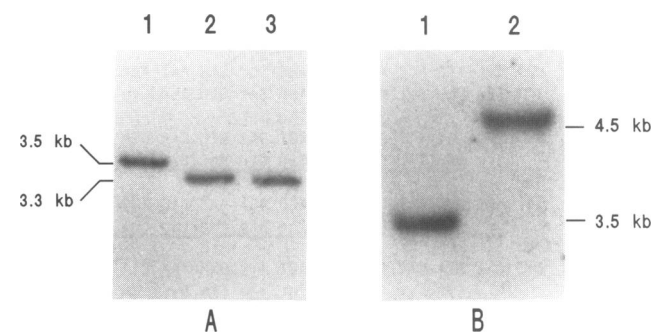

Fig. 1. Reverse phase HPLC separation of proteins of the small subunit of yeast mitochondrial ribosomes. Total small subunit proteins (500 μg in 250 μl 4% acetic acid) were injected onto a Vydac C18 column (218TP10: 10 μm particle size; 300 Å pore size; column dimension, 250×4.6 mm). Proteins were eluted at a flow rate of 0.5 ml/min with the following eluants: buffer A, 0.1% TFA in 10% acetonitrile; buffer B, 0.1% TFA in 50% acetonitrile. The gradient (dotted line) applied was: 0−20 mins, 10−15% B; 20−30 mins, 15−30% B; 30−65 mins, 30−32% B; 65−85 mins, 32−33% B; 85−105 mins, 33−33% B, 105−110 mins, 33−34% B; 110−130 mins, 34−34% B, 130−150 mins, 34−38% B, 150−180 mins, 38−38% B; 180−185 mins, 38−39% B; 185−195 mins, 39−40% B; 195−220 mins, 40−40% B; 220−230 mins, 40−45% B; 230−250 mins, 45−47% B; 250−270 mins, 47−55% B, 270−275 mins, 55−55% B, 275−280 mins, 55−10% B; 280−310 mins, 10−10% B. Proteins were monitored by absorbance at 214 nm. The inset is a blow up of the absorbance profile with retention times between 140 and 180 minutes. The shaded peak includes the MRPS28 protein.

Fig. 3. Genomic Southern hybridization of wild-type and disrupted copies of *MRPS28*. A) Genomic DNA was isolated from W303-1B cells, digested with: lane 1; EcoRI, Lane 2; BamHI and BglII, Lane 3; BglII, and hybridized with the MRPS28 oligonucleotide mixture at 60°C. B) Genomic DNA carrying either a 1) wild-type copy of *MRPS28* or 2) a disrupted copy of *MRPS28* was digested with EcoRI and hybridized with the MRPS28 oligonucleotide mixture at 60°C. The MRPS28 oligonucleotide mixture hybridizes to a 3.5 kb EcoRI fragment in W303-1B cells and to a 4.5 kb fragment in cells carrying a disrupted copy of *MRPS28*. The change in fragment size between these two strains is consistent with the disruption of chromosomal *MRPS28* outlined in Fig. 7 and described under 'Materials and Methods'.

mitochondrial, chloroplast, and eubacterial S15-like proteins shows greater than 33% sequence identity for each combination, with 19% of the sequence conserved in all three proteins. Given that mitochondria and chloroplasts are thought to have evolved from eubacterial ancestors via endosymbiosis (9,10), it seems reasonable to assume that these proteins are homologous. A
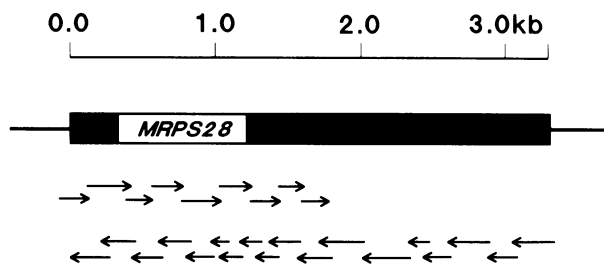

Fig. 4. Sequencing strategy for *MRPS28*. Serial deletions from both ends of the 3.3 kb fragment of yeast genomic DNA were made using Exo III and mung bean nuclease as described in 'Materials and Methods'. The DNA sequence was determined by the chain termination method of Sanger *et al.* (31). Solid horizontal lines refer to vector sequences. The shaded box refers to sequences 5' and 3' to *MRPS28* present in the 3.3 kb fragment. The open box refers to the *MRPS28* coding region. The arrows indicate the direction and boundaries of independent sequence determinations.

ribosomal protein from the archaebacterium, *H. marismortui*, has also been found to have sequence similarity with S15-like proteins. Allowing for the insertion of five amino acids toward the carboxy-terminus of the archaebacterial protein, there is 24%, 17%, and 16% sequence identity with eubacterial, chloroplast, and mitochondrial S15-like proteins, respectively. When all four S15-like sequences are aligned the sequence identity drops below 10% with only 7 positions conserved in all four proteins. One interpretation of these data is that the invariant amino acids play a critical role in some aspect of ribosome assembly and/or function that is conserved across wide phylogenetic boundaries. Our search of current data bases did not reveal a eukaryotic cytoplasmic ribosomal protein related to the S15-like proteins. It will be interesting to see if such a protein exists and if so, whether any of the conserved positions identified here extend to the eukaryotic kingdom.

Disruption of *MRPS28*

The chromosomal copy of *MRPS28* was disrupted according to Rothstein (40). The strategy for the construction of a plasmid where the bulk of the MRPS28 open reading frame is replaced by the yeast URA3 gene is outlined in Fig. 7. A linear fragment of DNA containing *URA3* and flanked by *MRPS28* sequences on both the 5' and 3' ends was used to transform the yeast strain W303-1B. Transformants were selected by uracil prototrophy

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-330 GATCTTCTAAGATATGTAATATTTCAAAGCTGTGGTTTGATTTTATGATCACAATATGTATCTATATATTGCTGATTGCATTTCAGCTTTTAACGAAAGTCCGCTATGCA

-220 AAACAGGTAACATAGCTGTAATACGTGAGCTCAACCAGCTTTGTTTGATCTTTTCTTATTTCGCCGTTCCACGGTTTTCCGTGATAGAGCAAGCAATATTGATTTGAAAA

-110 GAAAAATGAAAAGCGGTCAAGGTTAAAAACTTGCAATAAGGGTATTTAGCACTAGGTAGTCGCAGGTAAGCCTCTTAACACCACATTCAGGTATTTGAAGATAATACATA

  +1  ATG TCG ATT GTT GGA AGG AAT GCT ATT CTG AAT CTA AGA ATT TCA CTA TGT CCT CTG TTT ATG GGC AAA AGA TCG TTT GTA TCC
      MET Ser Ile Val Gly Arg Asn Ala Ile Leu Asn Leu Arg Ile Ser Leu Cys Pro Leu Phe MET Gly Lys Arg Ser Phe Val Ser

  85  TCT CCG GTT AGC AAT AGT GCA AAA GCT GTG AAA TTC TTA AAG GCT CAA AGA CGA AAA CAG AAA AAT GAA GCC AAA CAA GCC ACT
      Ser Pro Val Ser Asn Ser Ala Lys Ala Val Lys Phe Leu Lys Ala Gln Arg Arg Lys Gln Lys Asn Glu Ala Lys Gln Ala Thr

 169  TTG AAA GCG TCA ACC GAT AAG GTT GAT CCA GTT CTG GGC CGG GCG GAC ACT CCC TTT ATA ACA CGT ATT ATG GCA GAG TTA AAA
      Leu Lys Ala Ser Thr Asp Lys Val Asp Pro Val Leu Gly Arg Ala Asp Thr Pro Phe Ile Thr Arg Ile Met Ala Glu Leu Lys

 253  GAG CCT TTA GTT CTA TCG AAA GGT TAT AAT ATT GAA GAG GTA GAC AAG TTC CTT GCA GCC ATT GAG TCT GCT AAG CGT GAA AGA
      Glu Pro Leu Val Leu Ser Lys Gly Tyr Asn Ile Glu Glu Val Asp Lys Phe Leu Ala Ala Ile Glu Ser Ala Lys Arg Glu Arg

 337  GCT GAG TTG TCA GGT TTA AAT ACA GAA GTT GTG GGT ATT GAA GAC ATA GAA AAA CTG GAA GAC AGA CGT GAA GCC ATC TTG AGA
      Ala Glu Leu Ser Gly Leu Asn Thr Glu Val Val Gly Ile Glu Asp Ile Glu Lys Leu Glu Asp Arg Arg Glu Ala Ile Leu Arg

 421  ATA TTG AGC ATG AGG AAC TCC GAG AAT AAA AAT GCC ATT AAA ATG GCT GTA GAA CTC GCA CGT AAG GAA TTT GAG AGG TTC CCA
      Ile Leu Ser Met Arg Asn Ser Glu Asn Lys Asn Ala Ile Lys Met Ala Val Glu Leu Ala Arg Lys Glu Phe Glu Arg Phe Pro

 505  GGC GAT ACT GGC TCC AGT GAA GTC CAA GCA GCT TGC ATG ACT GTT CGT ATC CAG AAT ATG GCA AAC CAT ATT AAA GAG CAT CGT
      Gly Asp Thr Gly Ser Ser Glu Val Gln Ala Ala Cys Met Thr Val Arg Ile Gln Asn Met Ala Asn His Ile Lys Glu His Arg

 589  AAG GAT TTT GCT AAC ACC AGA AAT TTG AGG ATA TTA GTC CAG CAA AGG CAG GCA ATA TTA AGA TAT TTG AAA AGA GAT AAT CCT
      Lys Asp Phe Ala Asn Thr Arg Asn Leu Arg Ile Leu Val Gln Gln Arg Gln Ala Ile Leu Arg Tyr Leu Lys Arg Asp Asn Pro

 673  GAA AAA TAC TAC TGG ACT ATT CAA AAA CTA GGA TTG AAT GAT GCG GCC ATA ACG GAT GAA TTC AAT ATG GAC AGG CGT TAC ATG
      Glu Lys Tyr Tyr Trp Thr Ile Gln Lys Leu Gly Leu Asn Asp Ala Ala Ile Thr Asp Glu Phe Asn Met Asp Arg Arg Tyr Met

 757  CAA GAT TAT GAA TTT TTT GGT GAT AAA ATA TTG ATA AGA GAT TCT AAG AAA GTG GCA AAT CAA AAG CGT AAG GAG ATA AGA AAA
      Gln Asp Tyr Glu Phe Phe Gly Asp Lys Ile Leu Ile Arg Asp Ser Lys Lys Val Ala Asn Gln Lys Arg Lys Glu Ile Arg Lys

 841  CAA AAA AGG GCT ACA TTT TAG AATAATTTGAATCATATGTAGAAAAGCTTGTAAATAAGAAACCGTCATATTCTTTTTTTTTTTTTGACCGCGTATGAATCAGT
      Gln Lys Arg Ala Thr Phe TER

 945  AATTTATTTCATTTTGAATTACATCATTATAAAAAGTTCTTAGTAATATATGCATTGGGTATACAATCACGTGTACTATCAAATAAAGAAATGAAAATACGGATATACCTC

1056  TGTAATTAAGCCCCTAAGAGGGCTGTGTTTTCATCAGG---
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Fig. 5. Sequence of the MRPS28 gene. Shown below the DNA sequence is the amino acid sequence of a long open reading frame. Sequence obtained from the amino terminus of purified MRPS28p is found within the open reading frame from serine 34 to lysine 53 (underlined). The portion of the amino acid sequence homologous to ribosomal protein S15 from *E. coli* is underlined twice. Only sequence determined from both strands of the 3.3 kb yeast genomic fragment containing the MRPS28 open reading frame and portions of the 5' and 3' flanking region is reported.

and subsequently screened for growth on respiratory carbon sources. Genomic DNA was prepared from respiratory-deficient cells and Southern hybridization was used to show that the chromosomal copy of *MRPS28* in these cells was disrupted (Fig. 3). Myers *et al.* (41) have shown that mutations in components of the mitochondrial translation machinery that affect organellar protein synthesis cause these cells to spontaneously lose portions
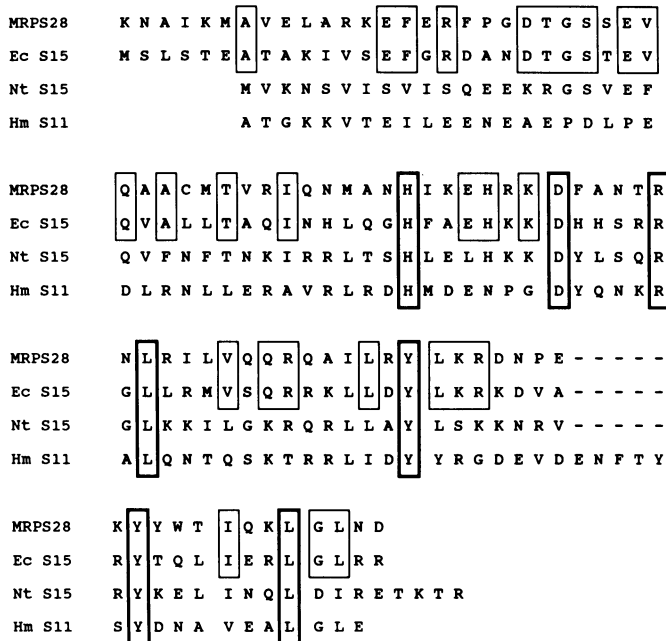

Fig 6. Sequence alignment of S15-like proteins. The alignments were made by the FASTA program of Pearson and Lipman (36). MRPS28, *Saccharomyces cerevisiae* mitochondrial ribosomal protein S28 ; Ec S15, *Escherichia coli* ribosomal protein S15; Nt S15, *Nicotiana tabacum* chloroplast ribosomal protein S15; and Hm S11, *Halobacterium marismortui* ribosomal protein S11. Boxed residues show identity between MRPS28 and Ec S15, residues boxed in bold type show identity between all four sequences. A five amino acid gap (dashes) is necessary for maximal alignment of MRPS28, Ec S15, and Nt S15 with Hm S11.
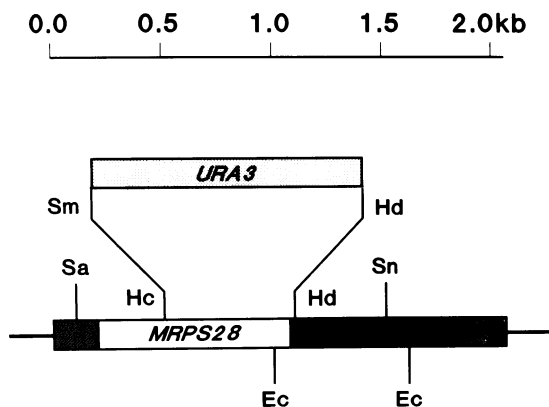

Fig. 7. Strategy for the disruption of *MRPS28*. Approximately 70% of the *MRPS28* coding region was replaced with the yeast URA3 gene. The disrupted gene was integrated into the yeast genome by homologous recombination where it replaced the chromosomal copy of *MRPS28*. Details of the gene disruption strategy are described under 'Materials and Methods'. Abbreviations for restriction enzyme recognition sites are: Sa, SacI; Sm, SmaI; Hc, HincII; Ec, EcoRI; Hd, HindIII; Sn, SnaBI. Results from the Southern hybridization showing the replacement of the chromosomal copy of *MRPS28* with the disrupted copy are shown in Fig. 3 B.

of their mitochondrial genomes at a high frequency. To test whether this occurred in cells carrying a disrupted copy of *MRPS28*, these cells were mated with a ρ° strain, COP161, with a wild-type copy of *MRPS28*. Diploids from this cross were tested for respiratory-competency by growth on YEPG media. After several generations of growth in non-selective media, cells carrying a disrupted copy of *MRPS28* were unable to form respiratory-competent diploids with the ρ° tester strain indicating that the disruption of *MRPS28* causes these cells to lose portions of their mitochondrial genomes at a high frequency. These data are consistent with an essential role for MRPS28 in mitochondrial protein synthesis.

DISCUSSION

We have purified a small subunit ribosomal protein from yeast mitochondria. Partial sequence information was used to isolate the nuclear gene, *MRPS28*, coding for this protein. Alignment of the amino acid sequence predicted from *MRPS28* with the amino acid sequence obtained directly from the purified protein showed that the MRPS28 protein is initially synthesized as a precursor with an amino-terminal extension that most likely serves as a mitochondrial targeting signal. Cells carrying a disrupted copy of *MRPS28* were unable to respire and spontaneously lost portions of their mitochondrial genomes at a high frequency, consistent with an essential role for MRPS28p in mitochondrial protein synthesis.

Sequence analysis of the MRPS28 gene revealed that a portion of the encoded protein was homologous to the *E. coli* ribosomal protein S15. The primary sequence conservation between MRPS28p and Ec S15 suggests that these two proteins have similar functional properties. One function of Ec S15 is its ability to bind to a stem-loop structure encompassing positions 654—751 of *E. coli* 16S rRNA (14—17). This stem-loop structure forms part of a minimal core structure common to virtually all known 16S rRNA-like sequences including that of yeast mitochondria (17). In addition to the conserved secondary structure in this region of 16S rRNA, some of the bases of the 717—730 loop that are protected from chemical attack upon Ec S15 binding are universally conserved and may represent important binding determinants for Ec S15. The structural conservation between MRPS28p, Ec S15, and other S15-related proteins may be a consequence of constraints on sequence divergence imposed by the need for these proteins to interact with highly conserved regions of rRNA.

Ec S15 is known to play a key role as an early assembly protein in the reconstitution of *E. coli* ribosomes *in vitro* (12). Despite these *in vitro* results, Ec S15 may not be essential for ribosome assembly and function under certain conditions *in vivo*. Yano and Yura (42) isolated an opal suppressor of the *rpoH11* mutation of *E. coli* that mapped to the Ec S15 gene. The Ec S15 gene in the suppressor strain has an insertion element in its 3' flanking region that reduces levels of Ec S15 mRNA and protein to nearly 10% that of wild-type. Polysomes isolated from the suppressor strain had substoichiometric amounts of the Ec S15 protein suggesting that functional ribosomes may be assembled in its absence. The suppressor strain was cold-sensitive, however, suggesting that the importance of Ec S15 in ribosome assembly and function may become more pronounced at lower temperatures. In contrast, MRPS28p appears to be essential for mitochondrial protein synthesis.

There are several potential explanations for the differences

between our results and those of Yano and Yura (42). The *MRPS28* disruption is effectively a null mutation since virtually all of the *MRPS28* coding region including the sequences homologous to Ec S15 is deleted. The suppressor strain of Yano and Yura, on the other hand, still retained a small amount of Ec S15 which could conceivably play a catalytic role in ribosome assembly. The phenotype of a null mutation in the Ec S15 gene has not been examined. Another explanation for why MRPS28p appears essential for mitochondrial protein synthesis *in vivo*, may be related to the lower growth temperatures used for yeast compared to *E. coli*. The suppressor strain of Yura and Yano is cold-sensitive, suggesting that subribosomal particles lacking Ec S15 are unable to overcome a temperature-dependent barrier in ribosome assembly. The normal temperatures for culturing yeast, which fall between 28 and 30°C, may represent non-permissive growth conditions for a strain lacking a functional mitochondrial counterpart to Ec S15. We have examined growth of cells carrying a disrupted copy of *MRPS28* on respiratory carbon sources at 34 and 37°C and found that cells were still unable to respire (data not shown). These data indicate that the requirement for MRPS28p in the assembly and/or function of mitochondrial ribosomes can not be circumvented by an increase in temperature and therefore that the *MRPS28* disruption is not mimicking the cold-sensitive phenotype of the *E. coli* suppressor strain. Finally, since MRPS28p is approximately 3 times the size of Ec S15 it is possible that the extra sequences present in MRPS28p not homologous to Ec S15 may play an essential role in mitochondrial protein synthesis.

MRPS28 is one of three nuclear genes coding for mitochondrial ribosomal proteins that contain regions of similarity with an *E. coli* ribosomal protein within the context of a much larger protein. The other two genes are *MRP7* (4) and *MRP-L20* (6). It has been suggested that these genes may have been created by the fusion of two smaller genes coding for proteins with different functions (4,6). One proposal suggests that as genes from the original mitochondrial genome were transferred to the nucleus, some were integrated into existing genes to form new hybrid genes. It is unclear at this point whether or not the function of the preexisting nuclear gene was lost after the putative integration event. If the function of the preexisting gene product was retained, the hybrid proteins so produced may be bifunctional. If, on the other hand the function of the preexisting gene was lost, its sequences may have been recruited to play an integral role in the function of the new hybrid protein. Finally, the sequences derived from the preexisting gene, other than the mitochondrial targeting signal, may not have a role in the function of the hybrid protein, in which case they should be dispensable.

MRPS28p, MRP7p, and MRP-L20p each appear to have retained an essential function of a eubacterial ribosomal protein within the context of a new hybrid protein. In the case of MRPS28p and MRP-L20p, these essential functions may be minimally defined by their abilities to interact with small and large subunit rRNAs, respectively, at early stages of subunit assembly. While these roles in subunit assembly may bear some similarities to their eubacterial counterparts, extra sequences present in these proteins may have roles in ribosome assembly and/or function more specific to mitochondrial ribosomes. The wealth of information available concerning the RNA binding properties of Ec S15 and its role in the assembly of *E. coli* ribosomes provides us with clear directions for further studies directed at a better understanding of the functional role of MRPS28p in the assembly and function of the mitochondrial

ribosome. These studies should not only provide us with a better understanding of the structure/function relationships of certain ribosomal proteins in the context of their general roles in protein synthesis in both eubacterial and mitochondrial ribosomes but also how these proteins have been adapted to function in association with other components of the mitochondrial ribosome.

ACKNOWLEDGEMENTS

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