Biochemical and Biophysical Research Communications **266,** 141–146 (1999)
Article ID bbrc.1999.1785, available online at http://www.idealibrary.com on IDEAL®

Identification of a Mammalian Mitochondrial Homolog of Ribosomal Protein S7

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Received November 1, 1999

Bovine mitochondrial small subunit ribosomal proteins were separated by two-dimensional electrophoresis. The region containing the most basic protein(s) was excised and the protein(s) present subjected to in-gel digestion with trypsin. Electrospray tandem mass spectrometry was used to provide sequence information on some of the peptide products. Searches of the human EST database using the sequence of the longest peptide analyzed indicated that this peptide was from the mammalian mitochondrial homolog of prokaryotic ribosomal protein S7 (MRP S7$_{human}$). MRP S7$_{human}$ is a 28-kDa protein with a p*I* of 10. Significant homology to bacterial S7 is observed especially in the C-terminal half of the protein. Surprisingly, MRP S7$_{human}$ shows less homology to the corresponding mitochondrial proteins from plants and fungi than to bacterial S7.

Key Words: **protein synthesis; ribosome; ribosomal protein; mitochondria; mammals.**

Protein synthesis in mammalian mitochondria has a number of interesting features that are not observed in the corresponding systems from prokaryotes or the cell cytoplasm (1). The ribosomes present in mammalian mitochondria are 55–60 S particles and are composed of small (28 S) and a large (39 S) subunits (2). The mechanism of polypeptide chain initiation in mammalian mitochondria is not understood. Mitochondrial 28 S subunits have an intrinsic ability to bind mRNAs in a sequence independent manner. This interaction may represent one of the early steps in chain initiation in this organelle (3–5).

Analysis of the protein composition of mammalian mitochondrial ribosomes indicates that they have 85–90 proteins (6, 7). This number is significantly higher than that observed in bacterial ribosomes and may exceed the number of proteins present in eukaryotic cytoplasmic ribosomes (8, 9). During the past several years a combination of protein sequencing and EST database searches has allowed the identification of 11 ribosomal proteins present in mammalian mitochondria. Nine of these are present in the large subunit while two are in the 28 S subunit (10–13).

In the present work, we report the identification of a mammalian small subunit ribosomal protein (MRP S7) that is the homolog of bacterial ribosomal protein S7. This protein is located in the head of the 30 S subunit in prokaryotic ribosomes and is thought to play an essential role in organizing the 3′ domain of the 16 S rRNA in the vicinity of the P- and A-sites.

MATERIALS AND METHODS

Preparation of bovine mitochondrial ribosomal proteins for 2D-gel electrophoresis. Bovine mitochondria and 28S subunits were prepared as described previously by Matthews *et al.* (6). The 28 S subunits were collected by centrifugation at 48,000 rpm for 6 h in a Beckman Type-50 rotor. The pellet (4.7A_{260}, approximately 390 pmol (14)) was resuspended in isoelectric focusing gel buffer containing 9.5 M urea, 2% Triton X-100, 2% ampholytes (consisting of 1.6% (v/v) pH 5-7 and 0.4% (v/v) pH 3-10), and 0.24 M 2-mercaptoethanol. The sample was clarified by centrifugation in a Beckman Airfuge at 35,000 rpm for 20 min prior to loading on non-equilibrium pH gradient tube gels as described by Cahill *et al.* (7). Following electrophoresis in the first dimension, gels were equilibrated in buffer (10% glycerol, 2% SDS, 1% dithiothreitol, 62.5 mM Tris-HCl, pH 6.8) and subjected to electrophoresis in the second dimension on 12% SDS–PAGE gels (15). Gels were stained with Pro-Blue stain (Owl Separation Systems Inc.) according to the manufacturer's protocol except that 5% acetic acid instead of 12% trichloroacetic acid was used as the fixing reagent.

Peptide sequencing by mass spectrometry. A band that was a good candidate for an mRNA binding protein from the small subunit was excised and digested in-gel with trypsin (Boehringer Mannheim, Indianapolis, IN) in 10 mM Tris-HCl, pH 8.0, according to the procedure of Shevchenko *et al.* (16) except that alkylation of sulfhydryls was accomplished with 4-vinylpyridine.

Liquid chromatography/mass spectrometric (LC/MS) analysis of

This work has been supported by funds provided by the National Institutes of Health (Grant GM32734).

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0006-291X/99 $30.00

the in-gel digest was done using an ultimate capillary LC system (LC Packings, San Francisco, CA) coupled to a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK) with a Z-spray ion source. Fifty μL of the digest was preconcentrated and desalted onto a 300 μm id $\times$ 1 mm guard column (LC Packings) packed with Pepmap C18 material using the Famos autosampler. Peptides were then separated using a 75 μm id $\times$ 15 cm capillary column packed with 3 μm Pepmap C18 material. Mobile phase A consisted of 0.1% formic acid in a 2% acetonitrile solution while mobile phase B consisted of 0.1% formic acid in a 80/20 acetonitrile/water solution. Peptides were eluted from the column into the microelectrospray ion source of the Q-TOF using a gradient of 1% B to 40% B in 30 min. The outlet of the capillary column was coupled to a platinum-coated fused silica spray tip (20 μm tip id outlet, New Objective, Inc., Cambridge, MA) which made electrical contact through the Picotip holder (New Objective) in the Z-spray ion source. MS survey scans were acquired at a rate of 2 per second from m/z 400–2000. The instrument was operated in a data-dependent MS to MS/MS switching mode where peptide ions detected in MS survey scans triggered a switch to MS/MS for obtaining peptide fragmentation spectra. Because no matches were obtained with the Mascot searches (Matrix Sciences, Ltd., London, UK), *de novo* peptide sequencing of the MS/MS-derived peptide fragmentation spectra was done using the PepSeq program (Micromass) or by manual analysis.

Computational analysis. The peptide sequences obtained were searched against the nonredundant protein database using the FASTA algorithm (17). EST database searches of the sequenced peptide fragments were performed using the BLAST search program (18). Sequence analysis and homology comparisons were done using the GCG DNA analysis software package (Wisconsin Package Version 10, 1999, Genetics Computer Group (GCG), Madison WI) and the results were displayed using BOXSHADE (version 3.21, written by K. Hofmann and M. Baron). The secondary structure of MRP $S7_{human}$ was predicted using the PHD program (19). Prediction of the cleavage sites for the mitochondrial signal sequence was carried out using PSORT.

RESULTS

Identification of bovine mitochondrial ribosomal protein S7 by tandem mass spectrometry. The small subunit of mammalian mitochondrial ribosomes is capable of binding mRNAs strongly in the absence of auxiliary factors. As a first step toward understanding the protein components of these ribosomes responsible for this property, small subunit proteins from bovine mitochondrial ribosomes were separated by two-dimensional gel electrophoresis. RNA binding proteins might be expected to have elevated isoelectric points. Hence, proteins in the most basic region of the gel were of the greatest potential interest. It is difficult to assign the protein(s) in this region of the 2-D gel system used here to the designations provided by Matthews *et al.* (6) since the gel systems are different. However, the region of the gel system selected for analysis here is likely to contain one or more of the proteins designated S12–S16 by that group.

This region of the gel was excised and the protein(s) present were subjected to in-gel digestion using trypsin. After digestion, the resulting peptide mixture was analyzed by LC/MS with data-dependent MS to MS/MS switching. The MS/MS fragmentation of the largest tryptic peptide analyzed and the deduced primary amino acid sequence of this particular peptide are shown in Fig. 1.

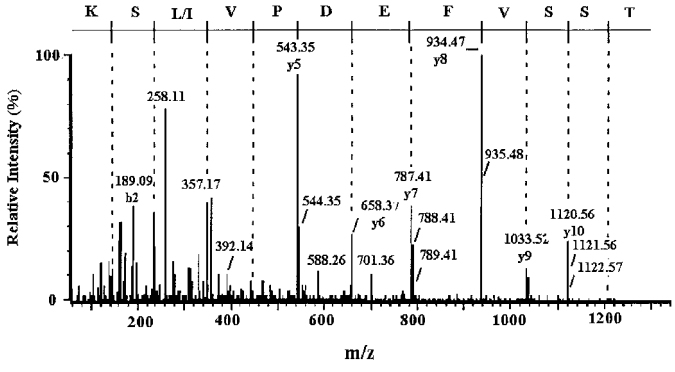

FIG. 1. The MS/MS fragmentation spectra of the peptide at *m/z* 654. 5 obtained from the tryptic digest of a mitochondrial small subunit protein isolated from the basic region of the 2-D gel. The sequence above the spectra indicates the amino acid sequence deduced from the fragmentation pattern.

Characterization of human mitochondrial ribosomal protein S7 from cDNA clones. The sequence of the peptide (Fig. 1) was used to search the human EST database using the tBLASTN program (National Center for Biotechnology Information). A number of hits were obtained. Most of these contained a perfect match to the peptide used as input. These clones presumably reflect sequencing of an overlapping region of the same cDNA in multiple clones. However, none of these clones had been identified as a specific protein (e.g clone H54652). After a refined search, a clone (AF077042) that had been identified as a human homolog of the bacterial ribosomal protein S7 was found. This clone contains a perfect match for the peptide sequence obtained from the bovine small subunit ribosomal proteins analyzed using mass spectrometry (Fig. 2). Based on this information, we propose that this clone encodes the mitochondrial homolog of S7. This protein will be referred to here as MRP $S7_{human}$ until it is feasible to correlate it with the nomenclature used previously (6).

The MRP $S7_{human}$ cDNA sequence reported in the EST database has 1046 nucleotides. The first AUG is located 182 residues from the 5′ end of the mRNA and is followed by an in-frame stop codon at position 911. No polyadenylation signal is apparent near the 3′ end of the cDNA sequence. Hence, it is likely that some information from the 3′ untranslated leader of this mRNA is not yet available.

The protein encoded in the open reading frame is 242 amino acids in length (Fig. 2). This protein has a molecular weight of 28,162 Da and a p*I* of 10.0. The N-terminal sequence was analyzed for the presence of a mitochondrial import sequence. Cleavage of the mitochondrial import signal on MRP $S7_{human}$ is predicted to occur at residue 37 using the PSORT program. The sequence at this site fits into the R2-cleavage motif

FIG. 2. Alignment of the deduced mammalian MRP S7 amino acid sequences with the bovine peptide sequence obtained by MS and with the corresponding proteins from mouse and rat EST databases (Table 1). (|) identical amino acids; (:) conservative amino acid replacements; (.) weakly conservative amino acid replacements; (*) partial sequence. The down arrow indicates the position predicted to be cleaved from MRP S7 following import into mitochondria.

(SRY↓SP) described previously (20). This region of MRP $S7_{human}$, thus, conforms well to typical mammalian mitochondrial signal sequences. The mature protein is predicted to be 205 residues in length with a molecular weight of 24,346 Da and a p*I* of 9.65. These values are in good agreement with the estimated values from the 2D-gel spot analyzed by mass spectrometry.

Using the MRP $S7_{human}$ sequence as a virtual screening probe, 4 hits were found in the mouse EST database and the complete sequence of MRP $S7_{mouse}$ was assembled (Table 1 and Fig. 2). MRP $S7_{mouse}$ is 241 residues in length, one residue shorter than the human sequence (Fig. 2). The difference in length is due to one additional amino acid in the import signal of the human protein. It should be noted that only one clone in the mouse EST covers this area of the cDNA, and due to a possible sequencing error, the clone had a frameshift at the 5′ end of the nucleotide sequence which was corrected manually. The import signal of MRP $S7_{mouse}$ is predicted to be 36 residues in length with cleavage occurring at the SRY↓SP sequence using the R-2 motif (20). Human and mouse MRP S7 align well over their entire length and show almost 85% identity (Fig. 2 and Table 2). Using the MRP $S7_{human}$ sequence as a virtual probe, 4 hits were also found in the rat EST database (Table 2). However, the complete sequence of the MRP $S7_{rat}$ could not be assembled (Fig. 2).

The primary sequence of MRP $S7_{human}$ has been compared to the sequences of its mitochondrial and bacterial counterparts (Fig. 3 and Table 2). If the predicted cleavage site of MRP $S7_{human}$ is correct, the mammalian mitochondrial protein is significantly longer (about 45–50 residues) than its prokaryotic counterparts which are generally 155–156 residues in length (Fig. 3). This difference in size is in agreement with the mobility of MRP $S7_{human}$ in SDS-PAGE. Most of the additional sequence predicted to be present in mature MRP $S7_{human}$ is located at the N-terminal end of the protein. This region of about 35 residues is highly charged with 10 acidic and 6 basic residues (Fig. 2). In general, bacterial S7s are 28–45% identical to MRP $S7_{human}$. The highest degree of identity observed to date is to the S7 from *Thermus thermophilus*. Interestingly, MRP $S7_{human}$ is no more closely related to the S7 of *Rickettsia* (the presumed progenitor of mitochondria) than it is to the S7 of other prokaryotes (Table 2). The conservation of these sequences is stronger in the

TABLE 1

Assembly of Identified EST Sequences from Rat and Mouse ESTs

Gene	bps nos.	Accession No.	Length ORF	Amino acids	
MRP-$S7_{human}$	183–911	AF077042	1046	1–726	1–242
MRP-$S7_{mouse}$	13–31	W11266	198	1–18	1–6
	7–441	AA474506	442	7–441	3–147
	423–3	AA014813	425	61–471	21–157
	513–34	AA024083	515	246–723	81–241
MRP-$S7_{rat}$*	328–176	AI176006	349	1–153	1–51
	20–265	H34589	267	96–279	33–93
	514–353	AI554989	514	387–549	129–183
	540–214	AA799673	542	399–726	134–242

Note. Construction of consensus cDNA sequences to obtain the full length mammalian MRP S7 ORFs by assembly of EST sequences. Consensus cDNA sequences are given in 5′ to 3′ direction. * Partial sequences of MRP-$S7_{rat}$ assembled by using the amino acid residue numbers of MRP-$S7_{human}$ gene as a reference. The designation "bps nos." indicates the base pair numbers in the cDNA translated to provide the indicated amino acid sequence.

TABLE 2

Percentage Identity of MRP $S7_{human}$ to S7 in Other Species

Species	% Identity	Evolutionary group
Mus musculus	84.6	Mammalian mitochondria
T. thermophilus	44.5	Thermophilic bacterium
E. coli	37.3	Purple nonsulfur bacteria, gram (−)
Rickettsia prowazekii	35.7	Purple nonsulfur bacteria
Bacillus subtilis	38	Low G + C, gram (+)
Euglena gracilis	30.5	Chloroplast
Synechocytis	29.7	Cyanobacteria
Marchantia polymorpha	23.1	Plant mitochondria
Wheat	29.3	Plant mitochondria
Arabidopsis thaliana	25.9	Plant mitochondria
Sulfolobus solfataricus	27.2	Archaea
Desulfurococcus mobilis	25.9	Archaea
Schizosaccharomyces pombe	22.6	Fungal mitochondria
S. cerevisiae	19.2	Fungal mitochondria
Human S5	28	Mammalian cytooplasm

Note. Percentage identity of S7 from *Rattus* sp. is obtained from an incomplete sequence.

C-terminal half than in the N-terminal half of the protein (Fig. 3).

Partial or complete sequences of MRP S7 from several plants (wheat, *Marchantia* and *Arabidopsis*) and from several fungi have been determined. The gene for MRP S7 is located in the mitochondrial genome of several plants (21) although it is a nuclear gene product in other plants and in yeast as it is in animal systems. Surprisingly, MRP $S7_{human}$ is generally less closely related to the other known MRP S7 sequences than it is to the bacterial and chloroplast S7 sequences (Table 2). The low homology is particularly apparent with yeast MRP S7 which is slightly less than 20% identical to the mammalian factor. The low percentage identity observed between the yeast and human MRP S7 has also been reported for the other mammalian mitochondrial ribosomal proteins (12).

The ribosomal protein equivalent to MRP S7 has been observed in the genomes of the archaea. These sequences are generally 25–30% identical to the mammalian mitochondrial protein (Table 2). From the perspective of sequence conservation, archael S7 is more closely related to the mammalian factors than is the fungal MRP S7. This observation calls into question the idea that the yeast mitochondrial translational system may serve as a model for mammalian mitochondrial protein synthesis. Cytoplasmic ribosomes also have a protein that has homology to bacterial S7. This protein (S5) has about 28% identity to MRP $S7_{human}$ (Table 2). Cytoplasmic S5 is shorter (204 amino acids) the MRP $S7_{human}$ but removal of the mitochondrial import signal should make the two proteins comparable in size.

DISCUSSION

The mechanism of translational initiation in mammalian mitochondria is poorly understood. Messenger RNAs are characterized by the presence of few, if any, nucleotides upstream of the start codon and, thus, do not have a Shine/Dalgarno sequence to direct initiation (22). These mRNAs are uncapped and do not use the cap binding and scanning mechanism for initiation found in the eukaryotic cell cytoplasm (23). But the small subunit of mammalian mitochondrial ribosomes possesses a strong, sequence-independent, affinity for mRNA (5, 24–26). Therefore, identification and characterization of small subunit ribosomal proteins that might interact with mRNAs could provide important insights into the mechanism of chain initiation. In the current report we have identified a protein in mammalian mitochondrial ribosomes that is the homolog of bacterial ribosomal protein S7.

In *E. coli* ribosomes, S7 is located in the head of the 30 S subunit and plays a major role in both initiation and elongation. It can be cross-linked to mRNAs just upstream of the start codon (27, 28) placing it in the

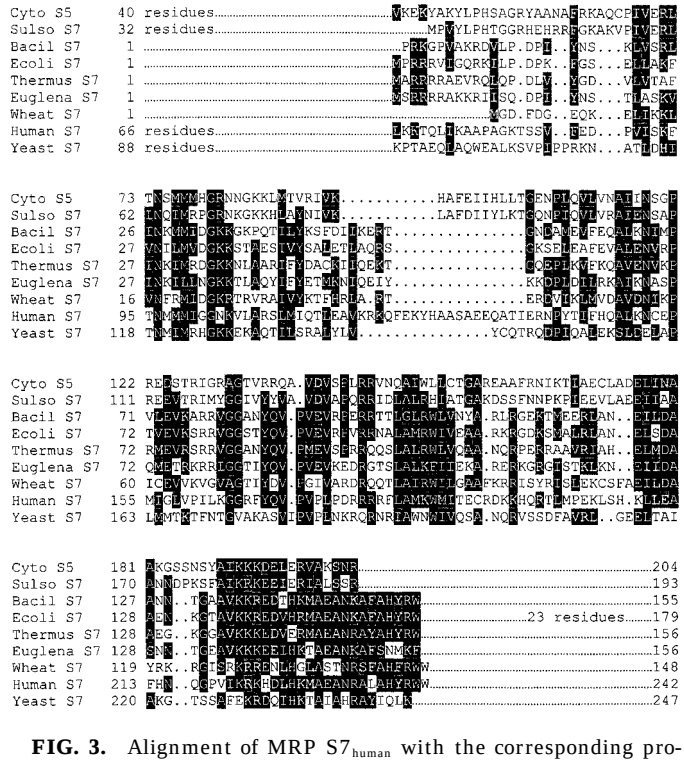

FIG. 3. Alignment of MRP $S7_{human}$ with the corresponding proteins from different species. Cyto S5, human cytoplasmic S5 from the 40 S subunit (belongs to the S7 family of ribosomal proteins); Sulso, *Sulfolobus solfataricus;* Bacil, *Bacillus subtilis;* Ecoli, *E. coli;* Thermus, *T. thermophilus;* Euglena, *Euglena gracilis* chloroplast; Wheat, *Triticum aestivum* MRP S7; Human, human MRP S7; Yeast, *Saccharomyces cerevisiae* MRP S7. Light shading indicates conservative amino acid replacements, and dark shading indicates identical amino acid residues.

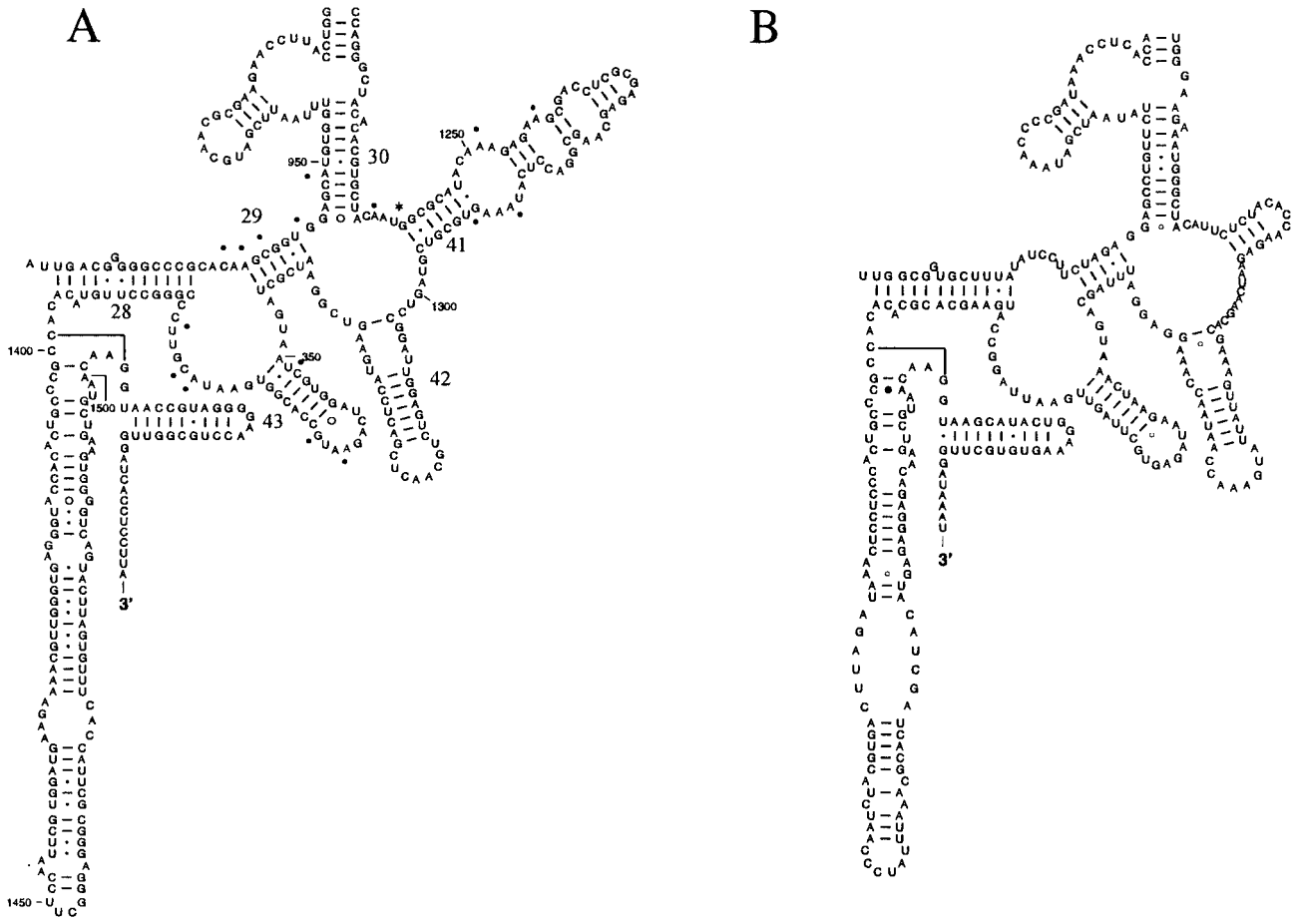

FIG. 4. Secondary structure of the S7 binding site in 16 S and 12 S rRNAs. (A) Region of the *E. coli* 16 S rRNA where S7 binds. Residues that have been shown to interact with S7 are indicated by a dot (●). The (*) indicates the residue that cross-links to Met114 of *E. coli* S7(36). Helix numbers are shown for those helices that cross-link to S7. (B) Corresponding region of bovine 12 S rRNA indicating that the major structural features present in the S7 binding site are present in the smaller organellar rRNA.

vicinity of the ribosomal P-site. Further, S7 can be cross-linked to tRNAs positioned within the A-site or the P-site (29, 30). S7 binds a defined region of the 16 S rRNA in the 3′ domain of the 16 S rRNA. It is referred to as a primary RNA binding protein of the small subunit and is thought to nucleate the folding of this half of the 16 S rRNA (31, 32). Major interactions between S7 and 16 S rRNA are observed in a number of loops and helices in this region (Fig. 4) (33, 34). The small subunit of mammalian mitochondrial ribosomes has a 12 S rRNA that is significantly shorter than bacterial 16 S rRNA. The 12 S rRNA shows little primary sequence conservation with the 16 S rRNA. However, many of the major secondary structural features have been preserved. The 12 S rRNA is not randomly shortened throughout its length but has certain major secondary structural elements amputated while others are preserved. Examination of the predicted structure of the 12 S rRNA in the vicinity of the S7 binding site indicates that the secondary structure in the proposed S7 binding site including helices 28, 29, 30, 41, and 42 have been conserved in the 12 S rRNA (Fig. 4) (35). *E. coli* S7 also interacts with helix 41 and the following loop (33, 34). This feature is present in a truncated form in the 12 S rRNA (Fig. 4). The conservation of the secondary structures of the 16 S and 12 S rRNAs makes it logical that mammalian mitochondria have an S7 homolog.

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