Identification of four proteins from the small subunit of the mammalian mitochondrial ribosome using a proteomics approach

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Abstract

Proteins in the small subunit of the mammalian mitochondrial ribosome were separated by two-dimensional polyacrylamide gel electrophoresis. Four individual proteins were subjected to in-gel Endoprotease Lys-C digestion. The sequences of selected proteolytic peptides were obtained by electrospray tandem mass spectrometry. Peptide sequences obtained from in-gel digestion of individual spots were used to screen human, mouse, and rat expressed sequence tag databases, and complete consensus cDNAs for these species were deduced in silico. The corresponding protein sequences were characterized by comparison to known ribosomal proteins in protein databases. Four different classes of mammalian mitochondrial small subunit ribosomal proteins were identified. Only two of these proteins have significant sequence similarities to ribosomal proteins from prokaryotes. These proteins are homologs to *Escherichia coli* S9 and S5 proteins. The presence of these newly identified mitochondrial ribosomal proteins are also investigated in the *Drosophila melanogaster*, *Caenorhabditis elegans*, and in the genomes of several fungi.

Keywords: Mitochondria; protein synthesis; ribosome; proteomics; mass spectrometry; ribosomal protein

Mammalian mitochondria carry out the synthesis of 13 polypeptides that are essential for oxidative phosphorylation and, hence, for the synthesis of the majority of the adenosine triphosphate (ATP) used by eukaryotic organisms. The ribosomes present in mammalian mitochondria are 55–60 S particles and are composed of small (28 S) and large (39 S) subunits. They are characterized by a low percentage of rRNA and a compensating increase in the number of ribosomal proteins (De Vries et al. 1973; O'Brien et al. 1976). The small subunit of the ribosome contains a 12 S rRNA and about 30 proteins, whereas the large subunit consists of a 16 S rRNA and about 50 proteins (Matthews et al. 1982). This total of about 80 proteins is significantly higher than that observed in bacterial ribosomes and may exceed the number of proteins present in eukaryotic cytoplasmic ribosomes (Wittmann-Liebold et al. 1980).

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Considerable progress has been made on the identification of the mitochondrial ribosomal proteins (MRPs) in yeast (*Saccharomyces cerevisiae*). About 50 different mitochondrial ribosomal proteins have been identified in this organism (Graack et al. 1998). Additional protein components in yeast mitochondrial ribosomes remain to be determined. Surprisingly, less than half of the MRPs in yeast show significant sequence identities to the ribosomal proteins of other systems (Graack and Wittmann-Liebold 1998).

Although the importance of the mitochondrial translation products is well known, limited information is available on the identities of the proteins that are parts of the translational machinery in mitochondria or on their relationships to bacterial ribosomal proteins. Recently, 18 proteins of the large subunit and 11 proteins of the small subunit of the mammalian mitochondrial ribosome have been characterized primarily by peptide sequencing coupled to the extensive use of the expressed sequence tag (EST) databases to deduce the full-length cDNAs and the corresponding amino acid sequences (Goldschmidt-Reisin et al. 1998; Graack et al. 1999; Koc et al. 1999, 2000; Mariottini et al. 1999;

O'Brien et al. 1999). Of these proteins, 12 from the large subunit and 4 from the small subunit are homologs of bacterial ribosomal proteins. The remainder fall into new classes of ribosomal proteins. In the current work, peptide sequence information has been obtained for four new mitochondrial small subunit ribosomal proteins. The cDNAs and amino acid sequences have been assembled using EST databases, and the genomes of *S. cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* have been searched for homologs. Only two out of the four new small subunit proteins found in mammalian mitochondrial ribosomes are similar to prokaryotic ribosomal proteins. The remaining two small subunit proteins fall into new classes of ribosomal proteins.

Results

Characterization of bovine MRPs by tandem mass spectrometry

As a first step toward understanding the protein components of mammalian mitochondrial ribosomes, small subunit proteins from bovine mitochondrial ribosomes were separated by two-dimensional polyacrylamide gel electrophoresis using the system developed by Cahill et al. with rat mitochondrial ribosomes (1995). Four individual protein spots that were well resolved and that had not been identified previously were selected for analysis (Fig. 1). These spots were excised, and the proteins present were subjected to in-gel digestion using endoprotease Lys-C. After digestion, the resulting peptide mixtures were analyzed by nanoscale capillary LC/MS/MS using a quadrupole time-of-flight mass spectrometer (Q-TOF) with data-dependent MS to MS/MS switching. As peptides are eluted from the nanoscale LC column and detected by the mass spectrometer, the mass spectrometer switches from MS to MS/MS mode in which product ion (fragmentation) spectra are obtained from each peptide to be used for determining the primary sequence of the peptide.

Each uninterpreted peptide product ion spectrum was initially searched against the nonredundant protein and EST databases for exact matches using the Mascot search program (Perkins et al. 1999). In some cases, high-quality product ion spectra that had no exact matches in the databases were sequenced de novo. A representative spectrum obtained from the protein designated as MRP-S27 and sequenced de novo is shown in Figure 2. Peptide sequences obtained from Mascot database searches and de novo sequencing are shown in Table 1.

Nomenclature

Previous work tentatively identified 33 proteins in the small subunit of bovine mitochondrial ribosomes (Matthews et al. 1982). These proteins were designated S1 to S33 in order of decreasing molecular weight. However, this nomenclature system does not provide a consistent set of designations for the MRPs from different mammals. For example, using this system, bovine MRP-S18 is the same protein as rat MRP-S13 (O'Brien et al. 2000). To simplify cross-species comparisons and to facilitate the correlation between proteins in mammalian mitochondrial ribosomes and in bacteria, we are using a nomenclature recently adopted for chloroplast ribosomal proteins (Yamaguchi and Subramanian 2000; Yamaguchi et al. 2000). In this system, proteins are designated by their prokaryotic homolog (e.g., S12 in bacteria is designated PRP-S12 in plastids and MRP-S12 in mammalian mitochondria). Proteins without bacterial homologs are given the next available number. Because there are 21 proteins in the bacterial ribosome, we began designating the new mammalian MRPs at MRP-S22 (Koc et al. 2000). The current manuscript describes two homologs of bacterial ribosomal proteins (MRP-S5 and MRP-S9) and two new small subunit ribosomal proteins (MRP-S27 and MRP-S28). The MRP-S28 that is described here is not related to yeast MRP-S28 (YDR337Wp) (Graack et al. 1998) or to the bovine MRP-S28 described previously (O'Brien et al. 2000).

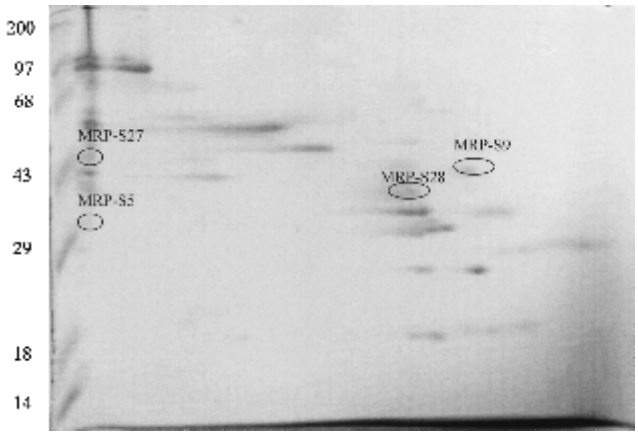

Fig. 1. Separation of bovine 28 S subunit proteins by two-dimensional gel electrophoresis using a NEPHGE-gel system developed previously (Cahill et al. 1995) for the separation of rat mitochondrial ribosomal proteins. The gel spots chosen for the in-gel tryptic digests are shown in ovals.

Overall approach to the assembly of mammalian MRP sequences from cDNA clones

Sequences of the peptides obtained from bovine mitochondrial ribosomes (Table 1) were used to search the human EST database using the tBLASTN program (National Center for Biotechnology Information). A number of hits were obtained for some, but not all, of the peptides used as virtual screening probes. Overlapping clones for these hits were obtained using the initial hits as virtual probes to rescreen the human EST database. For all the peptide sequences obtained, consensus cDNAs were assembled by repetitive searching and comparison of EST sequences. The sequence

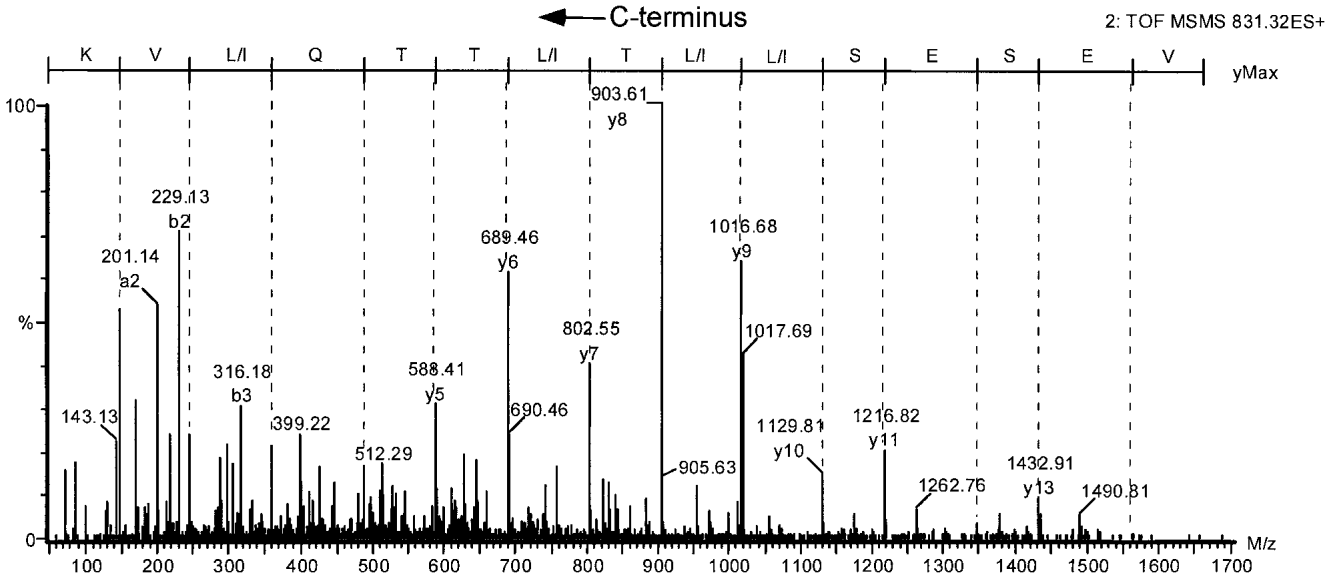

Fig. 2. Product ion spectrum of the tryptic peptide at m/z 831.8 from MRP-S27. Because there were no exact matches in the protein or EST databases, sequence was called de novo and is shown using the single letter amino acid symbols. Ions are labeled according to the nomenclature of Roepstorff and Fohlman (Roepstorff and Folhman 1984), with the difference in *m/z* of adjacent singly charged y ions corresponding to the labeled amino acid residue mass. For the isobaric amino acids leucine and isoleucine, both possibilities are shown as L/I.

of the longest possible cDNA was then assembled in silico. Sequencing errors were corrected as much as possible by comparison of overlapping clones. Generally, the fully assembled human sequence was then used as a query against entries in the mouse and rat ESTs. Mouse and rat consensus cDNAs were assembled for each protein when possible.

MRP- S5

A protein spot with a molecular weight of about 35 kD (see Fig. 1) was excised, and peptide sequence information was obtained as described above. Only one peptide sequence was obtained using this approach (Table 1). However, a second peptide sequence was obtained from the proteolytic digestion of whole 28 S ribosomal subunits followed by direct analysis by nanoscale capillary LC/MS/MS (E.C. Koc, K. Blackburn, W. Burkhart, A. Moseley, and L.L. Spremulli, in prep.; Table 1). Database searches with these peptides hit a number of EST clones described as similar to ribosomal protein S5 or MRP S5. Assembly of overlapping EST clones allowed the deduction of the complete sequence of human MRP-S5 (Fig. 3). The chromosomal gene for mitochondrial S5 has been located on human chromosome 5 (contig AC011416). It covers about 1.3 kb of DNA and consists of only one exon. Analysis of the sequence near the N-terminus of MRP-S5 for a mitochondrial import signal was performed using PSort, which predicts cleavage following residue 86. MitoProt II gives this protein a 78% chance of being mitochondrially localized and predicts cleavage following residue 87. The predicted mature form of human mitochondrial MRP-S5 has a calculated molecular mass of 39 kD, which corresponds well to the predicted molecular weight from the two-dimensional gel.

The sequence of human MRP-S5 was used to probe the mouse EST databases and allowed the deduction of the full-length sequence from this species. Mouse MRP-S5 is 79% identical to the human MRP-S5 (Table 2). The *C. elegans* genome has a homolog of human MRP-S5 (Swiss Prot Q93425). The *D. melanogaster* genome also has a putative MRP-S5 homolog. However, the full-length *D. melanogaster* homolog could not be obtained from the given sequences (AE003072 and EST AA438342). Alignment of the putative partial fly and human sequences indicates that they are closely related (Table 2).

The yeast mitochondrial ribosome also contains a homolog to prokaryotic S5 (Fig. 3; Table 2). Interestingly, the fungal MRP-S5 is no more closely related to mammalian MRP-S5 than are the bacterial proteins (Table 2). This trend has been observed with a number of the MRPs in yeast and mammals (Koc et al. 1999). Human MRP-S5 is considerably longer than prokaryotic S5 (Fig. 3; Table 2). There is a long extension at the N-terminus (about 180 residues) that is longer than the bacterial factors even after removal of the mitochondrial import signal. There is also a long C-terminal extension of more than 50 amino acids. These additional sequences also appear to be present in worm MRP-S5 (Fig. 3). They may play a role in interacting with some of the MRPs not present in prokaryotic and yeast mitochondrial ribosomes. The longer form of MRP-S5 found in mammalian mitochondrial ribosomes may also reflect the small rRNA present in the 28 S subunit or reflect basic differences

Table 1. *Amino acid sequences of mature MRPs of* Bos taurus *deduced from database searching of peptide product ion spectra of two-dimensional gel spots and whole 28S subunit*[a]

Protein name	Sequence	Swiss-Prot	Family
MRP-S5		P82675 (H)	S5
Peptide 1[a]	LTADELWK	P82674 (B)	
Peptide 2	...EPEDEVPD(L/I)Q...*		
MRP-S27		Q92552 (H) P82677 (B)	New
Peptide 1[a]	EDCHLADLASLMDK		
Peptide 2[a]	ALYTLVNK		
Peptide 3[a]	VQYGIFPDNYTFNLLMDHFIKK		
Peptide 4[a]	KTDFSWEERNNFGASLLLPGLK		
Peptide 5	...EQ(L/I)DVEETEQSK*		
Peptide 6	VESES(L/I)(L/I)T(L/I)TTQ(L/I)VK*		
MRP-S9		P82676 (B)	S9
Peptide 1[a]	RHLANMMGEDPETFTQEDVDRAITYLFPSGLFEK		
Peptide 2[a]	QRAVQWGEDGRPFHFLFYTGK		
Peptide 3	QSYYS(L/I)MHEAYGK*		
Peptide 4	EELEEMLVEK*		
Peptide 5	...IEP(L/I)QYDEQGMAFST...		
Peptide 6	QIEEFNIK		
MRP-S28		P82673 (H) P82672 (B)	New
Peptide 1[a]	EGNLELLK		
Peptide 2[a]	IPNFLHLTPVAIK (IPNFLHLTPVAIKK)		
Peptide 3[a]	LKNYCTEWPAALDSDEK		
Peptide 4[a]	LSSLNLDDHAK		
Peptide 5	(L/I)ET(L/I)FQ(L/I)K		

[a] Peptides are obtained from the digestion of whole 28S subunit by Endoprotease Lys-C. Molecular masses and pIs of proteins were determined from the predicted mature protein sequences by PSort from fully assembled cDNAs. (*) Sequence for these peptides obtained de novo, with (...) representing internally called sequence; (H) SwissProt number for the complete human sequence; and (B) SwissProt number for the bovine peptides.

in the mechanism of chain initiation in animal mitochondria compared with other systems.

MRP-S27

Peptide sequence information from this protein allowed deduction of the full coding sequence from data in the EST databases and GenBank from both humans and mice. Human MRP-S27 is 414 amino acids in length (Fig. 4). The mature protein is predicted to be 378 residues in length (MitoProt II) with a molecular mass of 43.8. Homologs of human MRP-S27 are detectable in *C. elegans* (CE16258) and, interestingly, in *Fugu rupripes* (puffer fish; AAC60297; Fig. 4; Table 2). The fish protein is 50.7% identical to human MRP-S27. The *D. melanogaster* genome has a region with a sequence similar to human and *C. elegans* MRP-S27. However, this predicted protein is only 22.5% identical to human MRP-S27 and is not in a predicted reading frame. No protein corresponding to human MRP-S27 can be detected in the yeast genome.

Analysis of the amino acid sequence of the MRP-S27 using PROSITE does not indicate the presence of any common motifs, including RNA binding or ribosomal protein motifs. However, MRP-S27 does possess one of three conserved blocks of sequence found in proteins that stimulate the dissociation of guanine nucleotides from G-proteins (Sone et al. 1997; Hoshino et al. 1999). Although this conservation is quite limited, the presence of a GTP-binding ribosomal protein in the small subunit of mammalian mitochondrial ribosomes (Denslow et al. 1991)[1] leaves open the possibility that MRP-S27 might be a functional partner of that GTP-binding ribosomal protein.

MRP-S9

Six peptides were obtained from a basic protein with a molecular mass of about 41 kD using mass spectrometry (Fig. 1; Table 1). Using the peptide sequences as virtual screening probes, the complete sequence of human MRP-S9 protein was assembled (Fig. 5). The MRP-S9 protein is 396 residues in length. The import signal of human MRP-S9

[1]Presence of a GTP-binding protein in the mammalian mitochondrial small subunit has been confirmed by the recent discovery of a putative GTP-binding protein (E.C. Koc, K. Blackburn, W. Burkhart, A. Moseley, and L.L. Spremulli, in prep.)

MRP-S5

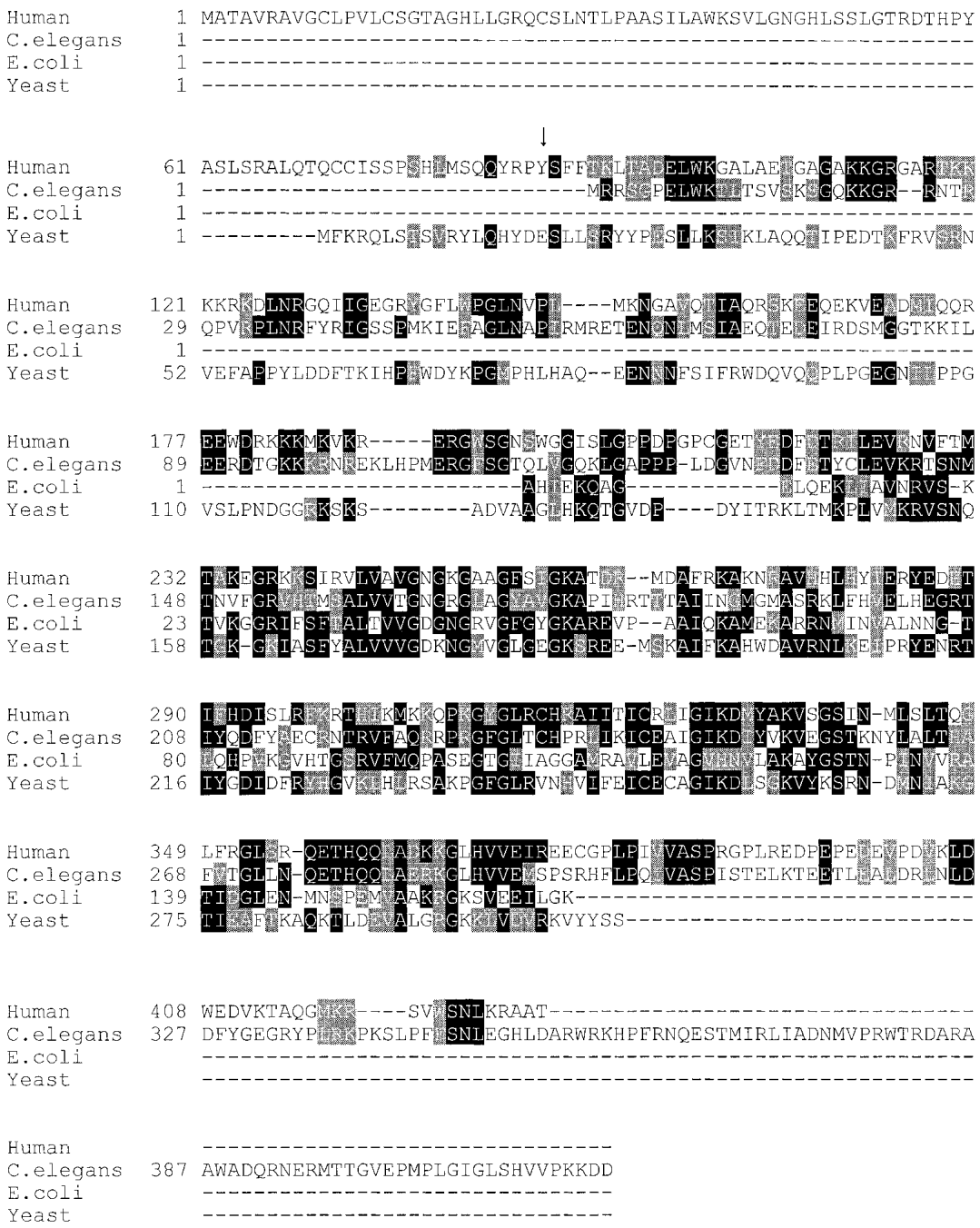

Fig. 3. Sequence of human MRP-S5 (P82675) and its alignment with homologs from *C. elegans* (Q93425), *E. coli* (P02356), and yeast (P33759, YBR251W or YBR1704). (↓) Shows the signal peptide cleavage site predicted by MitoProtII and, additionally, represents the predicted peptide signal cleavage sites in Figures 4, 5, and 6.

protein is predicted to be 52 residues in length with cleavage occurring at an R-2 motif as predicted by PSort and MitoProtII programs. Overall mouse and human MRP-S9 proteins are 78.8% identical (Table 2). However, the N terminus of MRP-S9 was not found in the mouse EST database. This sequence indicates that only a few residues of the import signal of mouse MRP-S9 are missing from the assembled sequence.

Table 2. *Percentage identity of human mitochondrial ribosomal proteins that are also found in other species*

Human protein	Percentage identity to				
	Mouse	*Drosophila*	*Caenorhabditis elegans*	Yeast	*Escherichia coli*
MRP-S5	79.0	46.5[a]	35.3	23.3	24.7
MRP-S27	77.1	22.5[b]	22.3	n.d.	n.d.
MRP-S9	78.8	33.4	36.5	33.0	38.6
MRP-S28	76.8	44.3	37.9	27.1	n.d.

(n.d.) Homologs of human ribosomal proteins are not detected in database searches.
[a] Only partial sequences could be found in the databases.
[b] Percentage identity is calculated by using a putative *Drosophila* homolog that is not found in the same frame as the predicted reading frame of AAF47311.

Proteins related to human and mouse MRP-S9 are present in *C. elegans*, *D. melanogaster*, and yeast (Fig. 5; Table 2). Analysis of the sequences obtained indicates that this protein falls into the S9 family of ribosomal proteins. However, in agreement with the size of MRP-S9 on the two-dimensional gel, the mammalian mitochondrial protein is considerably longer than its bacterial counterparts. Mammalian MRP-S9 begins to align with the bacterial proteins around residue 255 (Fig. 5). The alignment continues through to the C terminus. Interestingly, the *C. elegans* MRP-S9 appears to be even longer than the mammalian protein. The N-terminal half of MRP-S9 may be interacting with one or more of the ribosomal proteins in mitochondria that do not have prokaryotic homologs.

Yeast mitochondrial MRP-S9 (YBR146W or YBR1123) is 30.1% identical to the mouse protein (Table 2). It is intermediate in length between the mammalian and prokaryotic proteins (Fig. 5). Again, the C-terminal region of the yeast protein corresponds to bacterial S9 with additional residues located at the N terminus. The mitochondrial import sequence of yeast MRP-S9 is predicted to be only 11 residues in length. Hence, yeast MRP-S9 is probably about 100 residues longer than the bacterial proteins. The human cytoplasmic homolog of S9 (Rsp 16, P17008) also has considerable sequence identity (37.1%) to human MRP-S9 especially near the C-terminal end.

MRP-S28

In-gel digestion of a spot on the two-dimensional gel at a molecular weight of about 33.5 kD generated a single peptide. However, additional peptides from this protein were obtained from proteolytic digests of whole 28 S subunits (Table 1). The complete cDNA for this protein was found in the TIGR Tentative Human Consensus (THC) sequences and encodes a protein of 323 amino acid residues (Fig. 6). MitoProtII assigns a 95% probability that this protein is localized in mitochondria and predicts a cleavage position right after residue 30. Partial sequences for this protein were also found in the mouse and rat EST databases. Searches of the *C. elegans* and *D. melanogaster* databases also indicate homologs in these species (Table 2). A homolog of this protein was detected in the yeast genome using the *C. elegans* and *D. melanogaster* sequences as the query in advanced BLAST searches. Yeast MRP-S28 (NP_010460, YDR175Cp) is 319 aa residues in length and 27.1% identical to the human protein. The yeast MRP-S28 (YDR175Cp) described here was not previously identified as a ribosomal protein. MRP-S28 is the third representative of a MRP that is shared between mammals and yeast that is not represented in prokaryotic ribosomes.

Database searches did not reveal any proteins with known functions that correspond to MRP-S28, and no ribosomal proteins from prokaryotic organisms show significant similarities to it. Therefore, MRP-S28 represents a new class of ribosomal protein.

Discussion

The identification and characterization of the proteins present in mitochondrial ribosomes has been difficult because of their low abundance. During the past several years, significant progress has been made in identifying some of the proteins present by using a combination of protein sequencing and database searching. In the present report, two proteomics-based approaches consisting of either two-dimensional PAGE coupled with high-sensitivity peptide sequencing by mass spectrometry or direct peptide sequencing by mass spectrometry have been used to identify four different classes of mammalian mitochondrial ribosomal small subunit proteins that had not been characterized previously. This work increases the number of proteins identified in the small subunit to 15.

The small subunit of the mammalian mitochondrial ribosome is thought to have 33 different proteins based on two-dimensional PAGE. This number is considerably higher than the 21 proteins found in the 30S subunit of the prokaryotic ribosome (Wittmann 1986). Therefore, a number of the mammalian MRPs are not expected to be homologs of prokaryotic ribosomal proteins. Two of the four ribosomal proteins described in this paper and 4 of the previously characterized ribosomal proteins show significant sequence similarities to bacterial ribosomal proteins (Koc et al. 1999, 2000). These are S5, S7, S9, S10, S12, and S14.

Four of the six MRPs that have been identified having prokaryotic homologs (S7, S9, S10, and S14) are located in the head of the small subunit (Samaha et al. 1994). S9, the prokaryotic homolog of MRP-S9 identified in the current work, is classified as a tertiary rRNA-binding protein because its assembly into the small subunit is strongly dependent on the presence of other proteins rather than arising

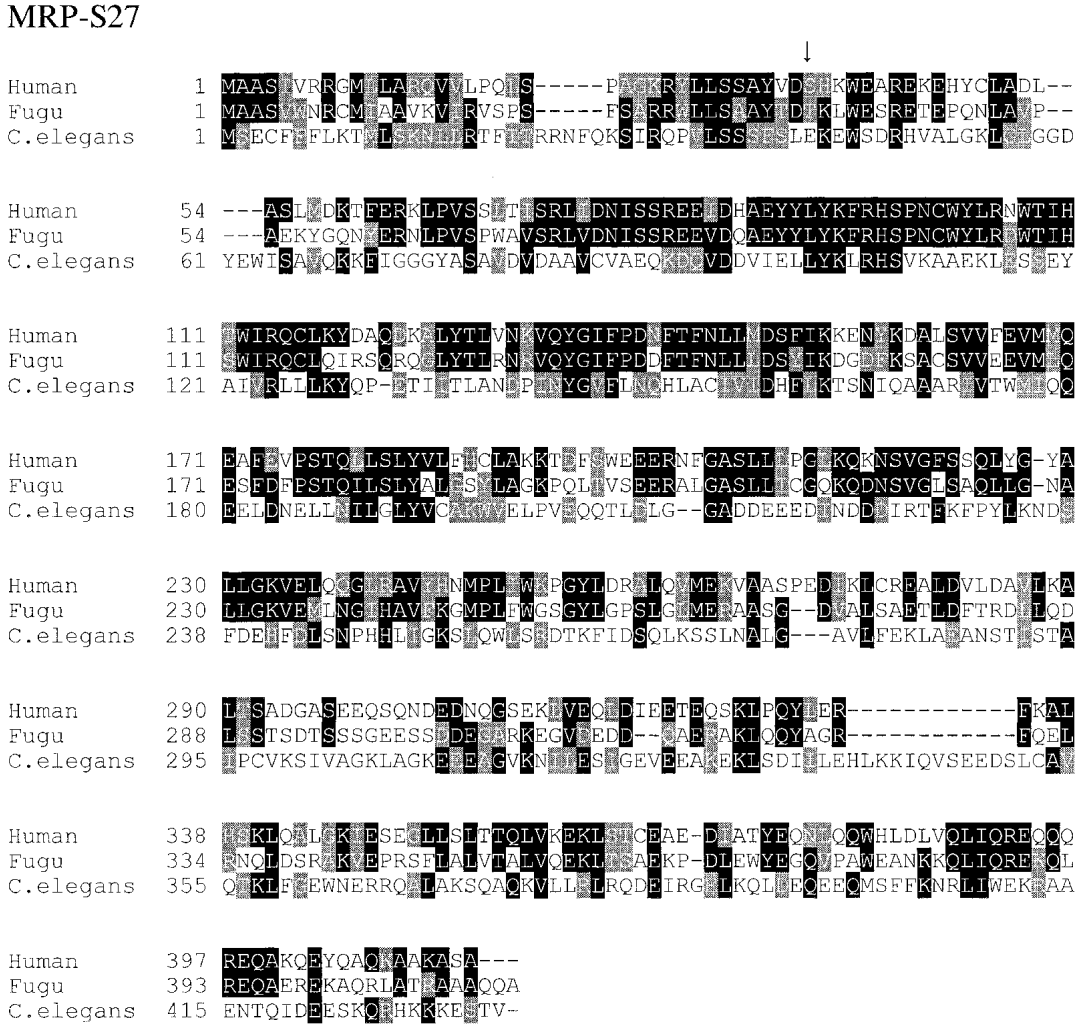

Fig. 4. Sequence of the human MRP-S27 (Q92552) and its alignment with homologs from *C. elegans* (CE16258) and *F. rubripes* (AAC60297).

from a tight direct interaction with the small subunit rRNA (Wittmann-Liebold 1985; Noller et al. 1990). Foot-printing and ultraviolet cross-linking experiments indicate that bacterial S9 is close to, or interacts with, helices 38, 39, and 41 in the 3′-domain of 16S rRNA (Samaha et al. 1994; Mueller et al. 1997). A comparison of the secondary structures of the *E. coli* 16 S rRNA and bovine mitochondrial 12 S rRNA indicates that the region corresponding to helix 39 is not present in the 12 S rRNA found in the 28 S subunit of the mammalian mitochondrial ribosome. This observation strongly indicates that S9 interacts primarily with other ribosomal proteins in the mitochondrial 28 S subunit. MRP-S9 is twice the size of bacterial S9 and has homology to the bacterial factor only in the C-terminal half of the protein. Sequences or domains in the N-terminal portion of MRP-S9 may play a role in interacting with some of the MRPs that do not have prokaryotic homologs. *E. coli* S9 can also be cross-linked to residue U60 in tRNAs bound to the A site of the ribosome (Abdurashidova et al. 1991). A similar proximity to the A site is likely for the C-terminal half of MRP-S9 that shows homology to the bacterial protein.

The present work has also identified a human mitochondrial protein in the S5 family of ribosomal proteins. A central region of MRP-S5 aligns with prokaryotic S5. Long N-terminal and C-terminal extensions surround the homology domain. In prokaryotic ribosomes, S5 is located in the body of the small subunit near a region involved in a conformational change during decoding (Lodmell et al. 1997; Clemons et al. 1999; Schluenzen et al. 2000). S5 is not among the six primary RNA-binding proteins in prokaryotes. It makes close contacts with both S4 and S8 which are primary rRNA-binding proteins. Although S5 is not a primary rRNA-binding protein, foot-printing and cross-linking experiments show that S5 is located near the pseudo-knot structure at the central domain of the small subunit around helix 27 (Heilek et al. 1996; Mueller and Brimacombe

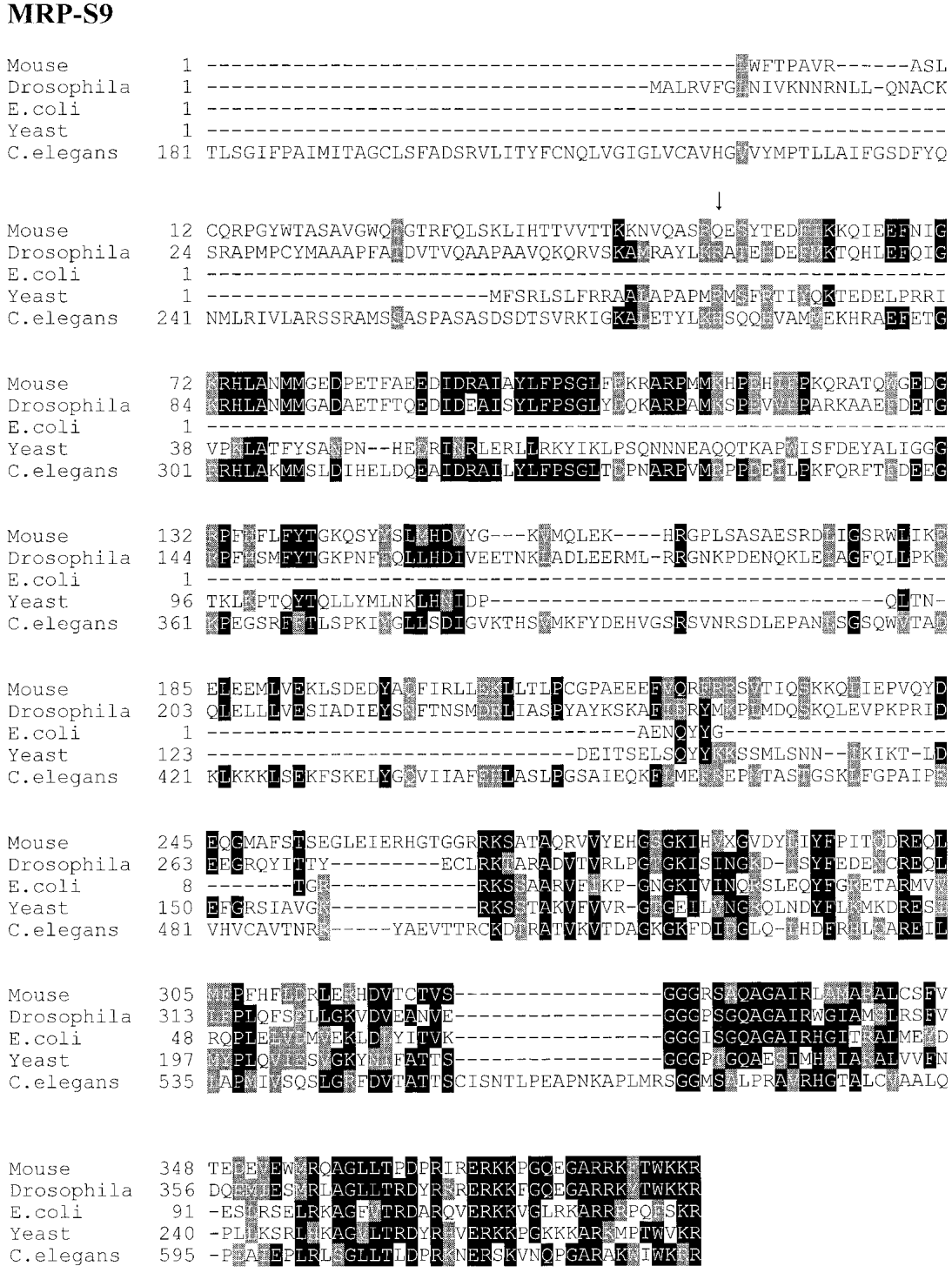

Fig. 5. Sequence of the human MRP-S9 and its alignment with homologs from *C. elegans* (P34388), *D. melanogaster* (CG2957), yeast (P38120, YBR146W or YBR1123), and *E. coli* (P02363).

1997). This region has been conserved in the 12 S rRNA of mammalian mitochondria. The structure of *Bacillus stearothermophilus* S5 has been determined (Ramakrishnan et al. 1992; Davies et al. 1998). This protein folds into two domains that interact strongly with each other through a hydrophobic core formed by the packing of one strand of the β-sheet in domain 1 against two α-helices in domain 2. As expected, the overall fold of MRP-S5 is predicted to be

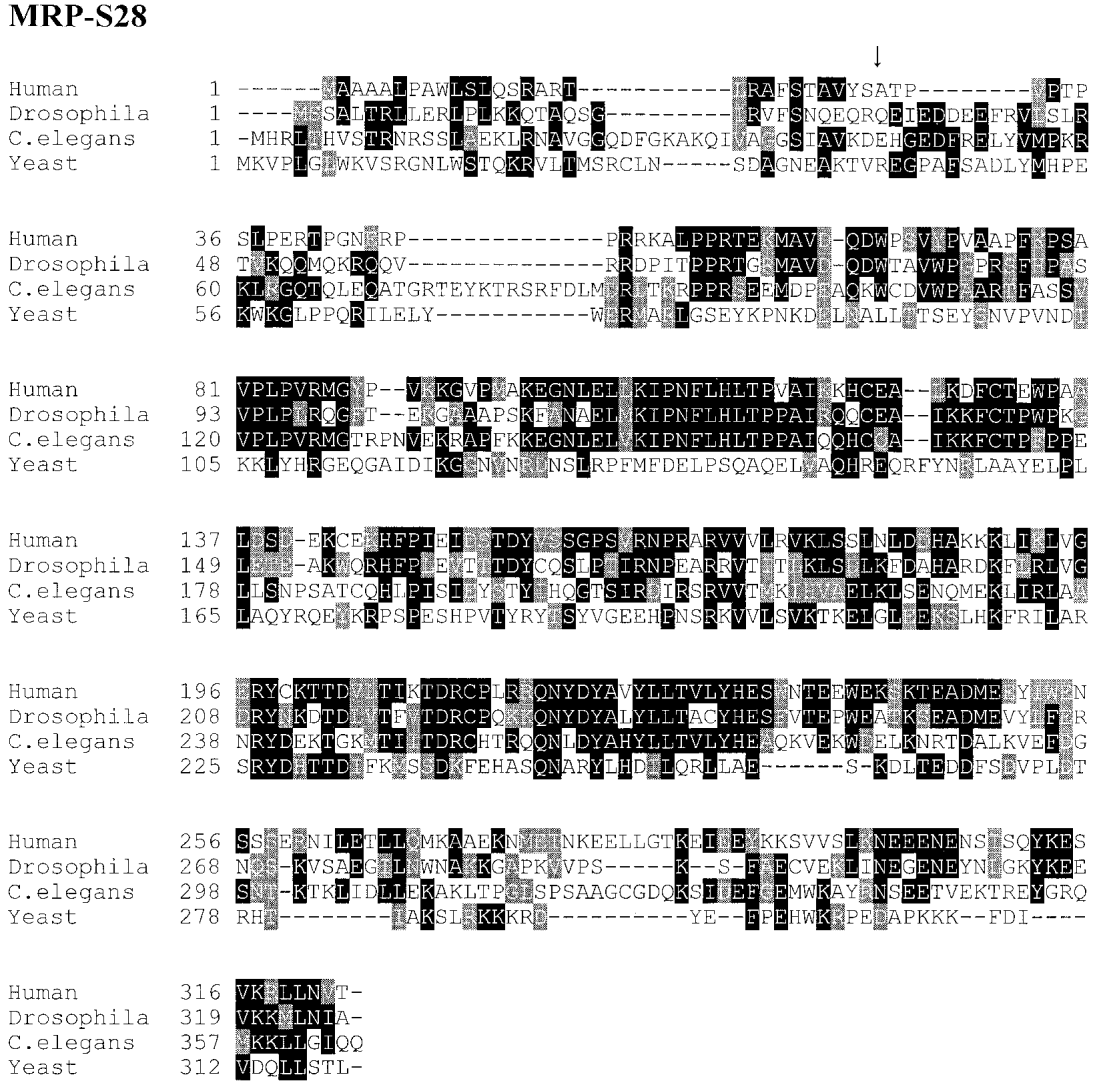

Fig. 6. Sequence of the human MRP-S28 (P82673) and its alignment with homologs from *D. melanogaster* (CG2101), *C. elegans* (CAB61063), and yeast (NP_010460 or YDR175Cp).

similar to that observed with prokaryotic S5 (Swiss Model). The residues forming the hydrophobic core are generally conserved or are conservative replacements. A series of Gly residues creating an overall pattern of turns in S5 are also generally conserved in MRP-S5. A surface loop rich in basic amino acids in the bacterial S5 is thought to interact with the 16 S rRNA. Mutations in this region are known to confer resistance to spectinomycin and are thought to be important for accurate translation. This region in MRP-S5 is modeled as a loop and is also rich in basic residues. Presumably, this region in MRP-S5 interacts with 12 S rRNA and may be important in maintaining ribosomal accuracy.

The two mammalian MRPs classified in new families identified in this study are not functionally characterized yet. Careful investigations of available databases identified homologs of these proteins in the yeast genome, but not in prokaryotic genomes. The presence of homologs of these two proteins in yeast brings yeast and mammalian mitochondrial translational systems closer to each other. Determination of the location and function of small subunit ribosomal proteins that are not homologs of known prokaryotic ribosomal proteins will require an extensive investigation of the structure of the mammalian mitochondrial ribosome.

Materials and methods

Preparation of bovine MRPs for two-dimensional gel electrophoresis

Bovine mitochondria and 28 S subunits were prepared as described previously by Matthews et al. (1982), and the 28 S subunits were collected by centrifugation at 48,000 rpm for 6 h in a Beckman

Type-50 rotor. The pellet containing about 5 A_{260} (~420 pmole; Hamilton et al. 1974) 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 prepared as described previously before loading on nonequilibrium pH gradient tube gels (Cahill et al. 1995; Koc et al. 1999). Following electrophoresis in the first dimension, gels were equilibrated in buffer (10% glycerol, 2% sodium dodecyl sulfate, 1% dithiothreitol, 62.5 mM Tris-HCl pH 6.8) and subjected to electrophoresis in the second dimension on 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE; Laemmli 1970). Gels were stained with Coomassie Brilliant Blue G-250 to locate protein bands.

Peptide sequencing by mass spectrometry

Four randomly picked spots from the two-dimensional PAGE of the mitochondrial ribosomal small subunit (28 S) were excised and digested in-gel with Endoprotease Lys-C (Boehringer Mannheim) in 10 mM Tris-HCl pH 8.0 according to the procedure of Shevchenko et al. (1996), except that sulfhydryl groups were alkylated using 4-vinylpyridine. Nanoscale capillary liquid chromatography-tandem mass spectrometric (LC/MS/MS) analyses of in-gel digests were performed using an Ultimate capillary LC system (LC Packings) coupled to a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass) fitted with a Z-spray ion source as described previously (Koc et al. 1999).

Additionally, 3 pmole of the whole 28 S subunit prepared by the traditional method (Matthews et al. 1982) were subjected to in situ endoprotease Lys C digestion to identify the mitochondrial small subunit ribosomal proteins in the intact subunit (Burkhart 1992). The peptides that were obtained from the whole 28 S subunit digests were also analyzed by LC/MS/MS (E.C. Koc, W. Burkhart, K. Blackburn, A. Moseley, and L.L. Spremulli, in prep.; a preliminary report was presented at the American Society of Mass Spectrometry in June 2000). Uninterpreted peptide product ion spectra generated by LC/MS/MS were searched against the nonredundant protein database and human and rat EST databases for exact matches using the Mascot search program (Perkins et al. 1999). High quality spectra that had no exact matches in either the protein or EST databases were sequenced de novo either manually or with the aid of the PepSeq program (Micromass).

Computational analysis

Peptide sequences obtained from Mascot searches of the protein and EST databases and those obtained by de novo sequencing from peptide product ion spectra were searched against the nonredundant protein database using the FASTA algorithm (Pearson et al. 1988). For peptides with no exact matches in the databases, sequences obtained by de novo sequencing were used for FASTA searches. Because mass spectrometry cannot distinguish between the isobaric (same nominal residue molecular weight) amino acids Leu and Ile, initial database searches were performed using Leu in the peptide sequences. Hits with an Ile at these positions were considered exact matches. If no hits were obtained when Leu was present in the search sequence, the search was reperformed with Ile. The isobaric amino acids Phe and oxidized Met (a common artifact of PAGE) were distinguished by diagnostic loss of methanesulfenic acid (64 Da) from oxidized Met (Jiang et al. 1996). Because the protease endoprotease Lys C, which cleaves on the C-terminal side of Lys residues, was used for in-gel and in situ digestions, Lys residues could be distinguished with a fairly high certainty from isobaric Gln residues. EST database and genomic DNA searches of the peptide sequences were performed using the BLAST search program (Altschul et al. 1997). Sequence analysis and homology comparisons were performed using the GCG DNA analysis software package (Wisconsin Package Version 10, Genetics Computer Group [GCG]), Vector NTI (Informax Inc.), and Biology WorkBench 3.2. The results were displayed using BOXSHADE (version 3.21, written by K. Hofmann and M. Baron). Prediction of the cleavage sites for the mitochondrial signal sequence was performed using PSort and MitoProt II (Nakai et al. 1992; Claros et al. 1996).

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