Toward the Full Set of Human Mitochondrial Aminoacyl-tRNA Synthetases: Characterization of AspRS and TyrRS†

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ABSTRACT: The human mitochondrion possesses a translational machinery devoted to the synthesis of 13 proteins. While the required tRNAs and rRNAs are produced by transcription of the mitochondrial genome, all other factors needed for protein synthesis are synthesized in the cytosol and imported. This is the case for aminoacyl-tRNA synthetases, the enzymes which esterify their cognate tRNA with the specific amino acid. The genes for the full set of cytosolic aaRSs are well defined, but only nine genes for mitochondrial synthetases are known. Here we describe the genes for human mitochondrial aspartyl- and tyrosyl-tRNA synthetases and the initial characterization of the enzymes. Both belong to the expected class of synthetases, have a dimeric organization, and aminoacylate *Escherichia coli* tRNAs as well as in vitro transcribed human mitochondrial tRNAs. Genes for the remaining missing synthetases were also found with the exception of glutaminyl-tRNA synthetase. Their sequence analysis confirms and further extends the view that, except for lysyl- and glycyl-tRNA synthetases, human mitochondrial and cytosolic enzymes are coded by two different sets of genes.

Aminoacyl-tRNA synthetases (aaRSs)[1] are key enzymes in the translation of the genetic information since they catalyze the specific attachment of each of the 20 amino acids (aa) to a cognate transfer RNA (tRNA). These enzymes have been studied equally well both at the gene and at the protein levels with most information gained on eubacterial, archæal, and eukaryal cytosolic (cyt) synthetases (reviewed in, e.g., *1*−*3*). From a structural point of view, despite the fact that they all fulfill the same function, the 20 enzymes are partitioned into two distinct structural classes at the level of their catalytic sites with 10 enzymes in each class (*4*, *5*). Class I enzymes bear the classical Rossmann fold (defined as an adenylic-nucleotides recognition site) that displays five parallel β-strands connected via α-helices and the two signature motifs HIGH and KMSKS. Class II enzyme catalytic sites display an alternate folding, mainly constituted by a sheet of six antiparallel β-strands and three motifs of less-conserved sequences (...**P**... for motif 1, ...F**R**XE... for motif 2, and ...GXGXGXE**R**... for motif 3). From a functional point of view, rules governing specific recognition by aaRSs of their cognate amino acids (*6*) and tRNAs (*7*−*9*) become well understood. From a cellular point of view, several sets of aaRSs have to coexist in the same eukaryotic cell to achieve protein synthesis within both cytosol and organelles. These three aspects raise questions on the distinguishing characteristics of mitochondrial enzymes and on the evolutionary history of their genes.

For mammals, and more specifically for humans, the genes for the 20 cytosolic enzymes are known, but limited information on mitochondrial aaRSs (mt-aaRSs) is available (*10*). The human mitochondrial genome itself codes for only 13 proteins (all subunits of the inner mitochondrial membrane respiratory chain complexes), 2 rRNAs, and 22 tRNAs (*11*), so that mt-aaRSs are necessarily nuclearly encoded and imported.

Most of the human mitochondrial aminoacylation activities were detected long ago, but genomic information on enzymes became available only recently. To our knowledge, sequence information and basic structural data on genes are available for mt-GlyRS (*12*, *13*) and mt-HisRS (*14*, *15*). Further, the genes of mt-IleRS (*16*, *17*), mt-PheRS (*18*), mt-LysRS (*19*), mt-LeuRS (*20*), mt-TrpRS (*21*), mt-SerRS (*22*), and mt-MetRS (*23*) have been identified and cloned, and the corresponding proteins have been characterized. Most of these mitochondrial enzymes (HisRS, IleRS, LeuRS, MetRS, PheRS, SerRS, and TrpRSs) are coded by different genes than the corresponding cytosolic synthetases. Only 2 out of the 9 previously characterized mitochondrial enzymes are encoded by the same gene than the cytosolic enzyme. Cyt- and mt-GlyRSs are generated from two translation initiation sites on the same gene, leading to one enzyme with a mt-targeting signal and a second with cytosolic location (*12*,

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[1] Abbreviations: tRNA, transfer RNA; aaRS, aminoacyl-tRNA synthetase with aa for amino acid (for individual aaRS, aa is given in the three letter code, e.g., AspRS for aspartyl-tRNA synthetase); mt, mitochondrial; cyt, cytosolic; EMAP, endothelial-monocyte-activating polypeptide.

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13). In the case of LysRS, an alternative mRNA splicing pathway allows the insertion, or not, of the mt-targeting signal leading to two mature enzymes differing only by a few residues at their N-terminus (*19*).

To expand the knowledge on human mt-aaRSs, we report here the identification and cloning of the genes coding for mt-AspRS and mt-TyrRS as well as the initial characterization of the two enzymes. Aminoacylation systems specific for aspartate or tyrosine from numerous organisms have already been investigated biochemically and structurally (reviewed in *24, 25*). AspRSs are typical class II aaRSs (they belong to subclass IIb), with a conserved modular architecture composed of a C-terminal active site domain linked to an N-terminal anticodon-binding domain by a short hinge. This organization is retained in the three domains of life. However, eukaryal AspRSs are distinguished from the others by an N-terminal extension involved in tRNA binding (*26*) and/or multisynthetasic complex formation (*27, 28*). Eubacterial AspRSs have an insertion module within the active site domain and a C-terminal extension. TyrRSs belong to class I aaRSs but in an ambiguous way. They share the typical class I sequence motifs, but their oligomeric structure is dimeric (a typical class II aaRS characteristic), and their mode of cognate tRNA recognition is similar to that of class II aaRSs (e.g., *29, 30*). Each monomer of TyrRS contains an N-terminal Rossmann-fold catalytic domain followed by an anticodon-recognition domain, which is smaller in archæbacteria as compared to the other phylae (*31*). In humans, cyt-TyrRS can be split into two fragments with distinct cytokine activities (*32*).

In an attempt to uncover the full set of genes for human mt-aaRSs, we have further taken advantage of the recent throughputs in human genome sequencing, have extensively screened protein, EST (expressed sequence tag), cDNAs, and genomic databases, and have identified potential open reading frames for 8 of the 9 missing genes.

MATERIALS AND METHODS

Materials. Native $tRNA^{Asp}$ from *Escherichia coli* was a kind gift from D. Kern (Strasbourg), and native $tRNA^{Tyr}$ from *E. coli* was from Subriden (Rolling Bay, WA). Purified oligonucleotides were from Proligo (Boulder, CO). L-[^{3}H]-aspartic acid (37 Ci/mmol) and L-[^{3}H]tyrosine (49 Ci/mmol) were from Amersham (Sweden). Restriction enzymes (*Bam*HI, *Bgl*II, *Bst*NI, *Hin*dIII, and *Pst*I) and T4 polynucleotide kinase were from New England Biolabs (U.K.). T4 DNA ligase was from Qbiogen (France). T7 RNA polymerase was purified from an overproducing strain as described (*33*). Dynazyme EXT polymerase was from Finnzymes (Finland).

Cloning Procedures. The same method was used to clone the genes of human mt-AspRS and mt-TyrRS. Genes were amplified from a human cDNA bank (gift from F. Martin, Strasbourg) using Dynazyme EXT polymerase. Primers for PCR were designed with restriction sites at their 5′-ends. Fresh PCR products were directly used for cloning into pCR2.1 following the TA Cloning Kit protocol (Invitrogen, Carlsbad, CA). After transformation of One Shot TOP10 *E. coli* cells, plasmidic DNA was purified, sequenced, and digested with the corresponding restriction enzymes. The fragment containing the expected gene was purified by gel electrophoresis, eluted, and inserted into the expression vector pQE70 (Qiagen, Germany) leading to pQE70-mt-AspRS and pQE70-mt-TyrRS plasmids.

For pQE70-mt-AspRS, primers designed for the PCR were as follows: 5′-*AGATCT*GTTGTCCGGACCAACACATGTGGA-3′ (5′-primer) and 5′-*AGATCT*ATGAGCTCTTTCTGCTTTGGAGTC-3′ (3′-primer), both including a *Bgl*II restriction site. The PCR was performed for 2 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 45 s at 52 °C, and 2 min at 72 °C and finalized with 10 min at 72 °C. Fifty nanograms of cDNA matrix and 2 U of polymerase were used along with 1 μM of each primer. Correct orientation of the gene within the pQE70 expression vector was verified by restriction fragment mapping.

For pQE70-mt-TyrRS, primers designed for the PCR were 5′-*GGATCC*ACACTCGGGCGCTCAGGGGTTACTGG-3′ (5′-primer including a *Bam*HI restriction site) and 5′-*AGATCT*CAACTGAAGCCATTTTATAATG-3′ (3′-primer including a *Bgl*II restriction site). PCR conditions were 5 min at 95 °C followed by 25 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 70 °C and finalized with 10 min at 70 °C. Two micrograms of cDNA matrix and 2 U of polymerase were used along with 1 μM of each primer. Plasmid pCR2.1-mt-TyrRS DNA was digested with *Bam*HI and *Bgl*II restriction enzymes to release the *mt-TyrRS* gene containing fragment and digested further with *Pst*I to eliminate other fragments of the same size.

Expression of the Cloned Genes. TOP10 strains transformed by plasmids pQE70-mt-AspRS or pQE70-mt-TyrRS were grown in a 200 mL culture of LB medium containing 50 μg/mL ampicilin and 2% glucose overnight at 37 °C. After dilution to 1:10 with 1800 mL of fresh medium, cells were grown at 37 °C for 2 h until an OD_{600} of 0.7 was reached. The medium was then replaced by LB medium containing 50 μg/mL ampicilin and 500 or 10 μM IPTG, respectively, and the incubation continued overnight at 25 °C. The cells were harvested by centrifugation at 4000*g* for 10 min (4 °C).

Purification. TOP10-pQE70-mt-AspRS and TOP10-pQE70-mt-TyrRS cell pellets were resuspended in 45 mL of buffer A (50 mM NaH_2PO_4 pH 7.5, 300 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol, and 10% glycerol) before sonication (Ultrasons Annemasse, France) on ice at 120 V for 40 s, 7 times. Lysed cells were then subjected to centrifugation at 30 000*g* for 45 min at 4 °C. If necessary, the supernatant was filtered through a 0.45 μm MillexHA filter (Millipore, France) before loading onto a 2 mL Ni−NTA resin column (Qiagen) equilibrated in buffer A. After the column was washed with 50 mL of buffer A, mt-AspRS and mt-TyrRS were eluted with a 30 mL linear gradient of buffer A to B (buffer A adjusted to 500 mM imidazole). The enzyme-containing fractions, detected by the OD_{280} profile and a SDS−PAGE analysis, were pooled and dialyzed overnight at 4 °C against 1 L of buffer C (50 mM KH_2PO_4/K_2HPO_4 pH 7.5, 150 mM KCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and 50% glycerol) and stored at −20 °C. Protein concentrations were determined either from OD_{280} using the theoretical extinction coefficient calculated with ProtParam from Expasy tools ($\epsilon = 43\,540\ M^{-1}\cdot cm^{-1}$ for mt-AspRS and $\epsilon = 39\,520\ M^{-1}\cdot cm^{-1}$ for mt-TyrRS) or from the Bradford method (Bio-Rad) using bovine serum albumin as reference.

Analytical Procedures. Gel filtration chromatography was performed at 4 °C using a 15 mL Bio-Prep SE-100/17 column (Bio-Rad) equilibrated with buffer 1 (50 mM NaH_2PO_4 pH 7.5 and 300 mM NaCl) for mt-AspRS or buffer 2 (50 mM HEPES−NaOH pH 7.5, 10 mM $MgCl_2$, 150 mM KCl, 1 mM DTE, and 20% glycerol) for mt-TyrRS. The column was calibrated with a set of standard proteins (molecular weights of 1.35, 17, 44, 158, and 670 kDa, Bio-Rad). Enzyme samples of 200 μL [at 0.6 mg/mL for mt-AspRS and 2 mg/mL (HEPES−NaOH buffer) for mt-TyrRS] were applied to the column. Molecular weights (MW) were calculated from log(MW) versus elution volume plots.

Cloning and in Vitro Transcription of tRNAs. Clones containing genes for human mt-tRNA^{Asp} and mt-tRNA^{Tyr} were obtained by hybridization of nine and eight overlapping phosphorylated oligonucleotides, respectively, ligation into *Hin*dIII and *Bam*HI sites of plasmid pTFMA and pUC119, respectively, and transformation into *E. coli* TG1 cells. Both synthetic genes contain a T7 RNA polymerase promoter followed by a hammerhead ribozyme sequence and the tRNA sequence (*34*). A *Bst*NI site, coincidental with the 3′-end of the tRNA sequence, allowed the synthesis of tRNAs ending with the expected CCA-3′-sequence. Transcription was performed according to established procedures (*34*, *35*). The hammerhead ribozyme self-cleaves the phosphodiester linkage directly upstream nucleotide 1 and liberates the tRNA. After phenol extraction, the transcripts were purified on denaturing PAGE, electro-eluted, and ethanol-precipitated. The concentration of transcripts was determined spectrophotometrically with 40 (μg/mL)/cm for 1 OD_{260} unit.

Aminoacylation Assays. Aminoacylation assays were performed as described (*36*). Aspartylation and tyrosylation conditions were 50 mM HEPES−KOH pH 7.6, 25 mM KCl, 12 mM $MgCl_2$, 2.5 mM ATP, 0.2 mg/mL BSA, 1 mM spermine, 32 μM [^{3}H]aspartate (208 GBq/mmol) or 10 μM [^{3}H]tyrosine (3 TBq/mmol) respectively, and adequate amounts of transcripts (0.04−1.6 μM) and enzymes (10−150 nM). Transcripts were renatured by heating at 60 °C for 90 s in water and slow cooling to room temperature before the aminoacylation was performed at 25 °C (aspartylation) or 37 °C (tyrosylation). The lower temperature chosen for aspartylation was required to obtain reproducible results in aminocylation. It corresponds to a compromise between optimal enzymatic activity and tRNA stability as was previously shown for in vitro transcribed mt-tRNA^{Lys} (*35*). This is likely a more general situation since human mt-tRNAs are encoded by either of the two DNA strands of strongly biased nucleotide composition and thus are either "heavy" and stable (tRNA^{Tyr}) or "light" and thermodynamically weak (tRNA^{Asp}) (*37*). After different incubation times, aliquots were removed, spotted on Whatman 3MM paper, and precipitated in 5% trichloroacetic acid. Incorporation of radioactive amino acid was measured by liquid scintillation counting. Plateau levels of charging were 60% for mt-tRNA^{Asp} and 90% for mt-tRNA^{Tyr}. Kinetic parameters k_{cat} and K_M were derived from Lineweaver−Burk plots. Displayed data represent an average of three independent experiments.

In Silico Methods. Genes for human aaRSs have been searched in selected databases (Ensembl Human database, NCBI protein, EST, cDNAs, and genomic databases, and euGenes), using entire or partial sequences of known aaRSs (of a given specificity) from other organisms as queries. Programs were (i) standard nucleotide−nucleotide Blast (Blastn) which compares nucleotide sequence queries against the NCBI nucleotide databases, (ii) tBlastn, which compares a protein sequence against NCBI nucleotide database translated into all 6 reading frames, and (iii) the standard protein−protein Blasts, Blastp and Ballast. The first two methods lead to nucleic acid sequences or accession numbers (DNA, cDNA, or mRNA), and the third method leads to protein sequences or accession numbers.

Programs and Web sites are 3DCoffee (http://igs-server.cnrs-mrs.fr/Tcoffee/), Ballast (http://igbmc.u-strasbg.fr:8080/ballast.html), Blast (http://www.ncbi.nlm.nih.gov/blast/), BoxShade (http://www.ch.embnet.org/software/BOX_form.html), DbClustal (http://igbmc.u-strasbg.fr:8080/DbClustal/dbclustal.html), Ensembl Human database (http://www.ensembl.org/Homo_sapiens/), ESPript (http://prodes.toulouse.inra.fr/ESPript/ESPript/), euGenes (http://iubio.bio.indiana.edu:8089/), ExPASy (Expert Protein Analysis System) Proteomics tools (http://www.expasy.org/tools/), MitoProt (http://ihg.gsf.de/ihg/mitoprot.html), NCBI databases (http://www.ncbi.nlm.nih.gov/), Prediction servers at CBS (http://www.cbs.dtu.dk/services/), PredictProtein (http://www.embl-heidelberg.de/predictprotein/predictprotein.html), Predotar (http://genoplante-info.infobiogen.fr/predotar/predotar.html), and TargetP (http://www.cbs.dtu.dk/services/TargetP/).

RESULTS

In Silico Characterization of Mitochondrial Aspartyl- and Tyrosyl-tRNA Synthetases. Screening of genomic databases with a set of known synthetases of same specificity pinpointed reliable candidates for both human mt-AspRS and mt-TyrRS genes. Their respective gene names are *HSM804946* (nucleic acid accession number AL833633) and *FLJ13995* (nucleic acid accession number AK024057 and protein accession number BAB14806). The corresponding translated sequences, named herein mt-AspRS and mt-TyrRS, are displayed in Figures 1 and 2 (first lines). Arguments in support of the two sequences to be the expected enzymes are the following. Both candidate sequences hit with high scores a cluster of aaRSs of same specificity (a selection of these is presented in Figures 1 and 2). Further, catalytic site motifs of aaRSs (*4*, *5*) are present. For mt-TyrRS, HVGH and KLGKS consensus sequences mimic the class I specific HIGH and KMSKS sequences; for mt-AspRS, motif 1 (...**P**...), motif 2 (...Y**R**DE...), and motif 3 (...GGIAL-GLD**R**LICLV....) defining class II aaRSs are present. Additionally, both sequences contain an N-terminal mitochondrial-targeting sequence as supported by two predictive programs (Predotar and MitoProt). These programs calculate the N-terminal protein region that can support a mt-targeting sequence and predict possible cleavage sites for the tag after import into the mitochondria (see "predicted tag cleavage positions" in Figures 1 and 2). Probabilities to be mitochondria addressed were found as 0.816 and 0.998 for mt-AspRS and 0.944 and 0.974 for mt-TyrRS. These scores are indicative of mitochondrial sequences and allowed us to get rid of false positive candidate sequences which all had scores below 0.05.

The gene for human mt-AspRS codes for a total of 645 aa from which 47 are predicted to correspond to the mt-

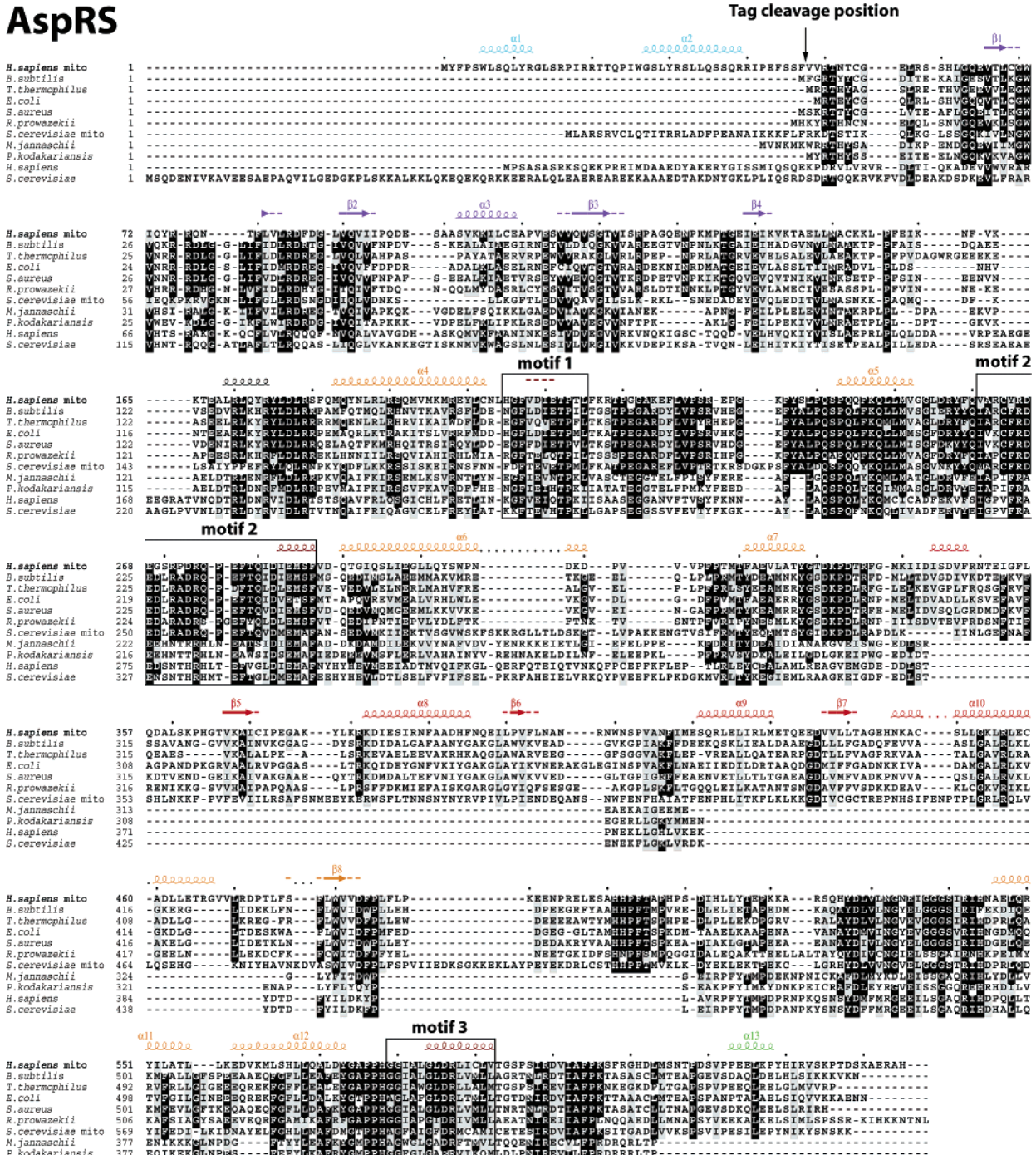

FIGURE 1: Sequence organization of human mitochondrial AspRS. Multiple sequences alignment of human mt-AspRS (in bold) with a selection of known AspRSs has been established using 3DCoffee program (*70*) and shaded with BoxShade. Notice that sequences have been reordered in accordance with their hit score toward human mt-AspRS. Black and gray boxes highlight, respectively, strictly identical and similar residues. Human mt-AspRS structure elements displayed above the sequence (α-helices and *β*-strands) have been predicted using PredictProtein (*71*) and drawn with ESPript (structure 2D) (*72*). For the color code, see Figure 5. The three signature motifs specifying class II aaRSs are framed. Accession numbers of sequences are *Bacillus subtilis*, O32038; *Thermus thermophilus*, P36419; *Escherichia coli*, P21889; *Staphylococcus aureus*, Q99TL9; *Rickettsia prowazekii*, Q9ZE17; *Saccharomyces cerevisiae* mt, NP_015221; *Thermococcus kodakaraensis*, Q52428; *Methanococcus jannaschii*, B64494; *Homo sapiens*, AAH00629; *Saccharomyces cerevisiae*, P04802. Cleavage position for the mitochondrial tag is indicated by an arrowhead.

targeting sequence, thus leading to a mature enzyme of 598 aa. Multiple alignment with related enzymes (Figure 1) reveals 36−43% identity of the full-length sequence with bacterial sequences, 32% with the mitochondrial sequence from the lower eukaryote *S. cerevisiae*, and below 23% with enzymes from archæa and cytosol of eukaryotes, including human cyt-AspRS. Alignment also shows that human mt-AspRS possesses strictly conserved residues found in all known AspRS sequences (*24*). These include residues involved in ATP binding and tRNA binding. Residues involved in amino acid binding include those typical for class II aaRSs and those specific for aspartic acid recognition. Further, the alignment illustrates the already known strong conservation of AspRSs from different phylae. Clusters of

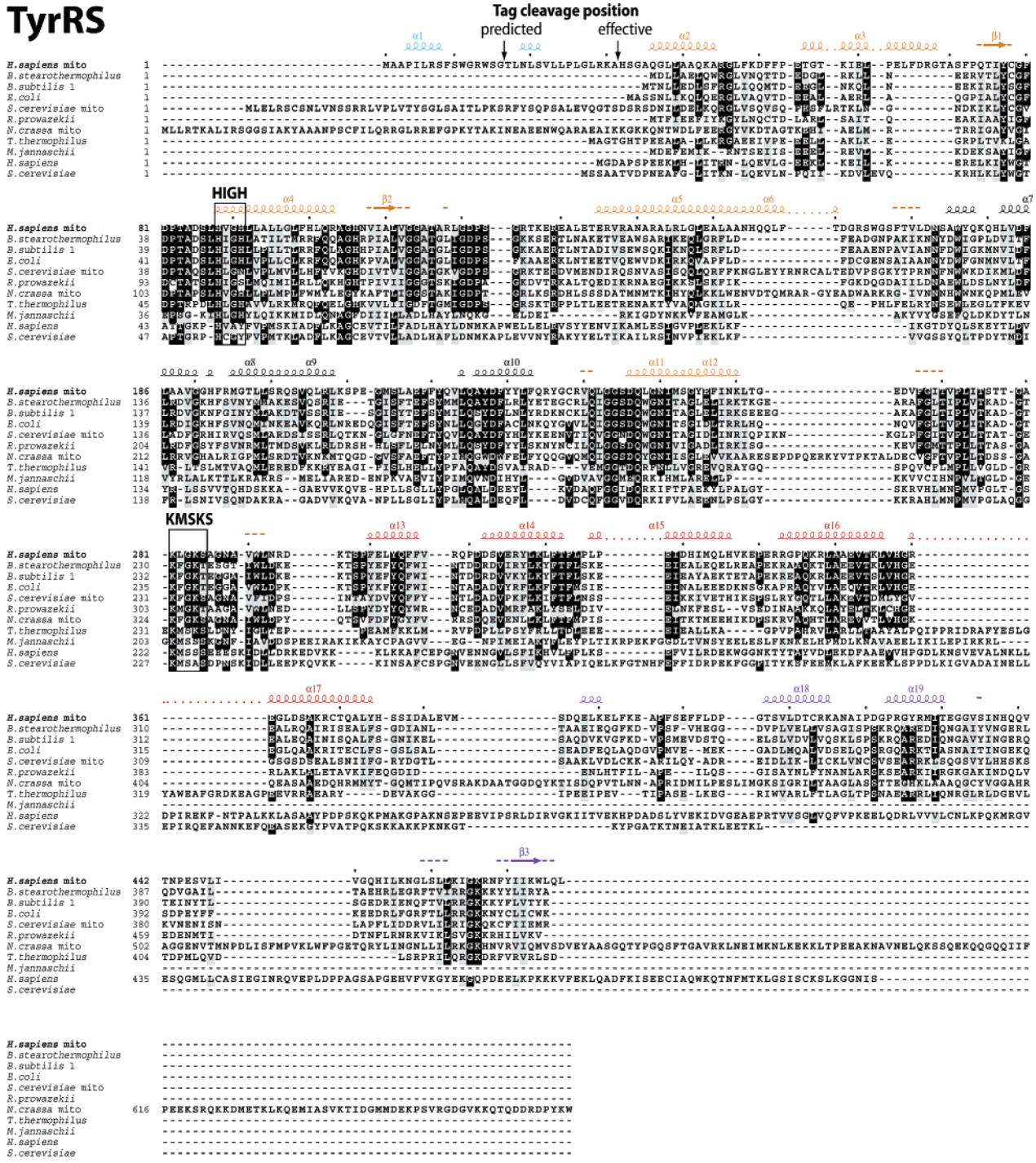

FIGURE 2: Sequence organization of human mitochondrial TyrRS. Multiple sequences alignment of human mt-TyrRS (in bold) with a selection of known TyrRSs has been established as for mt-AspRS (see legend of Figure 1 for details). The two signature motifs HIGH and KMSKS specifying class I aaRSs are framed. Accession numbers of sequences are *Bacillus stearothermophilus*, P00952; *Bacillus subtilis*, P22326; *Escherichia coli*, P00951; *Saccharomyces cerevisiae* mt, P48527; *Rickettsia prowazekii*, Q9ZCZ4; *Neurospora crassa* mt, P12063; *Thermus thermophilus*, P83453; *Methanococcus jannaschii*, Q57834; *Homo sapiens* cyt, P54577; *Saccharomyces cerevisiae* cyt, P36421. Predicted and effective tag cleavage positions (see text) are indicated by arrowheads.

strongly conserved amino acids are visible (black squares) all along the sequence except within domains corresponding to the eubacterial insertion (from residue 328 to 456) and C-terminal extension (from residue 607 to 645).

The gene for human mt-TyrRS encodes a protein of 477 aa including a predicted 16 amino acid long mt-tag (Figure 2, upper line). Alignments with known TyrRS sequences from all domains of life (of which some examples are shown in Figure 2) reveal 25−40% identity with a panel of eubacterial TyrRSs, 33 and 34% with yeast and *Neurospora crassa* mt-TyrRS sequences, and less than 25% with the enzymes from archæa and eukaryotes. Noticeable is the case of human cyt-TyrRS that presents no significant identity with its homologous mitochondrial enzyme. From this alignment, it further appears that the greatest homologies between all TyrRSs are exclusively contained within the N-termini, whereas C-terminal domains vary in size as well as in sequence. The very low sequence conservation is further

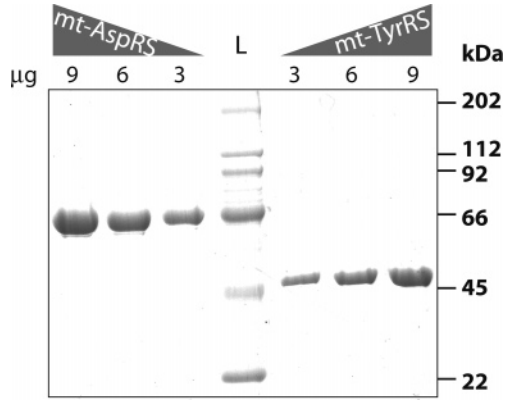

FIGURE 3: SDS−PAGE analysis of overexpressed and purified mt-AspRS and mt-TyRS. Samples of 3, 6, and 9 μg of enzyme preparations were analyzed on a 10% SDS−PAGE, and the protein bands were visualized by staining with Coomassie blue. Mt-AspRS is on the left side of the gel, and mt-TyrRS is on the right side of the gel. L stands for molecular weight ladder (standard mixture for molecular weights 22−202 kDa from Sigma).

illustrated by the difficulty to find residues conserved in all phylae. This is opposite to the case of AspRSs described above.

Biochemical Characterization of Human Mitochondrial Aspartyl- and Tyrosyl-tRNA Synthetases. The mature versions of the two enzymes were cloned into pQE70 vectors and expressed in *E. coli* strain TOP 10. As mentioned above, a theoretical cleavage site of the mt-targeting sequence can be predicted in silico for each enzyme. However, in the case of mt-TyrRS, we have designed a new cleavage site (see "effective tag cleavage position" in Figure 2). This was based on the fact that multiple alignments with bacterial TyrRS sequences suggest a larger targeting signal than that predicted in silico. Accordingly, a recombinant enzyme starting at position 32 (instead of 17), upstream a structurally predicted helix, was designed. As a result of the cloning procedure, additional residues were introduced at both ends of the molecule: four at the N-terminus and eight at the C-terminus (including six histidines). As a consequence, the recombinant human mt-TyrRS contains 458 aa, and its theoretical molecular weight is 51 302 Da. In the case of mt-AspRS, the mt-targeting sequence is of 47 aa, leading to a mature enzyme of 598 aa. For cloning purposes, six and eight aa (including six histidines) have been inserted at the N- and C-terminal parts, respectively. The recombinant human mt-AspRS contains 612 aa, with a theoretical molecular weight of 69 627 Da.

The two proteins were overexpressed in *E. coli* under similar conditions and purified by affinity chromatography on a nickel column. Human mt-TyrRS was eluted at 100 mM and mt-AspRS at 170 mM imidazole. The two proteins are pure to more than 95% as estimated on a Coomassie blue stained SDS−polyacrylamide gel (Figure 3). To be noticed is the low solubility of both enzymes in a variety of buffers (Tris-HCl, MES−NaOH, HEPES−NaOH, sodium cacodylate, potassium phosphate), and this at different pH and in a large range of ionic strength values. In short, mt-AspRS is only significantly soluble in phosphate buffers ($\leq$1 mg/mL), while mt-TyrRS is more soluble both in phosphate and in HEPES−NaOH buffers (to a maximal concentration of 2 and 4 mg/mL, respectively). Detailed data on solubility properties will be published elsewhere.

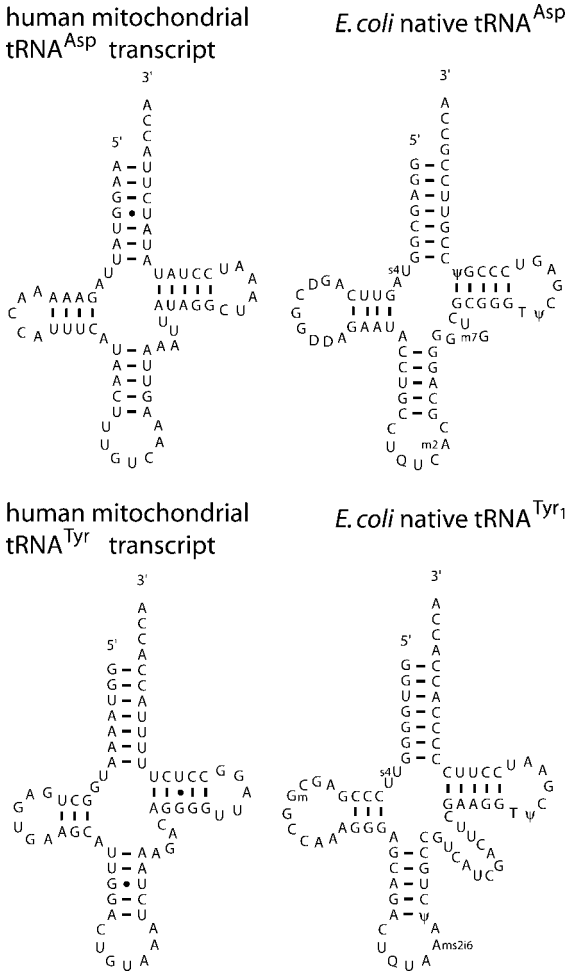

FIGURE 4: Secondary structures of tRNA^{Asp} and tRNA^{Tyr}, substrates of human mt-AspRS and mt-TyrRS, respectively. tRNAs are either in vitro transcripts based on human mt sequences (*73*) (left part) or native tRNAs from *E. coli* (right part). Names of modified bases are according to ref *74*.

Table 1: Kinetic Parameters for tRNA Aminoacylation by Human mt-AspRS and mt-TyrRS

tRNA	K_M (μM)	k_{cat} (10^{-3} s^{-1})	k_{cat}/K_M (10^{-3} s$^{-1}\cdot\mu$M^{-1})
mt-AspRS Enzyme			
E. coli native tRNA^{Asp}	0.15 ± 0.05	133 ± 23	887
human mt-tRNA^{Asp} transcript	0.13 ± 0.07	10 ± 3	77
mt-TyrRS Enzyme			
E. coli native tRNA^{Tyr}	0.52 ± 0.2	210 ± 70	400
human mt-tRNA^{Tyr} transcript	4.8 ± 1.2	46 ± 3	10

To evaluate the oligomeric status of both enzymes, gel filtration chromatographies were performed. Mt-AspRS and mt-TyrRS migrate with apparent molecular weights of 143 and 130 kDa, respectively, suggesting that they are dimers (results not shown). This is in agreement with their belonging to subclasses IIb and Ib, respectively, and does not depart from what was established for aaRSs of same specificities but from different organisms.

The capacity of both synthetases to aminoacylate tRNAs has been verified on purified *E. coli* tRNA^{Asp} and tRNA^{Tyr} as well as on in vitro transcripts of human mitochondrial tRNA^{Asp} and tRNA^{Tyr} (Figure 4, Table 1). *E. coli* tRNAs are classical substrates to test for mitochondrial activities and were shown to replace well the hardly accessible native

Table 2: Human Cytosolic and Mitochondrial Aminoacyl-tRNA Synthetases in Databases[a]

class I aaRSs					class II aaRSs				
aaRSs		gene name	nucl acc no.	prot acc no.	aaRSs		gene name	nucl acc no.	prot acc no.
ArgRS	cyt	*RARS*	NM_002887	NP_002878	***AlaRS***	cyt	*AARS*	NM_001605	NP_001596
	mt	*LOC57038*	NM_020320	NP_064716		***mt***	*LOC221410*	XM_291190	XP_291190
CysRS	cyt	*CARS*	NM_001751	NP_001742	***AsnRS***	cyt	*NARS*	NM_004539	NP_004530
	mt	*FLJ12118*	NM_024537	NP_078813		***mt***	*FLJ32418*	AK056980	
GlnRS	cyt	*QARS*	NM_005051	NP_005042	***AspRS***	cyt	*DARS*	NM_001349	NP_001340
	mt	not found	not found	not found		***mt***	*HSM804946*	AL833633	
GluRS	cyt	*EPRS*	NM_004446	NP_004437	**GlyRS**	cyt	*GARS*	NM_002047	NP_002038
	mt	*KIAA1970*	BC040013	AAH40013		**mt**	*GARS*	NM_002047	
IleRS	cyt	*IARS*	NM_013417	NP_038203	**HisRS**	cyt	*HARS*	NM_002109	NP_002100
	mt		D28500	BAA95147		**mt**	*HARSL*	NM_012208	NP_036340
LeuRS	cyt	*LARS*	D84223	BAA95667	**LysRS**	cyt	*KARS*	NM_005548	NP_005539
	mt	*LARS2*	NM_015340	NP_056155		**mt**		AF285758	AAG30114
MetRS	cyt	*MARS*	NM_004990	NP_004981	**PheRS**	cyt α	*FARSL*	NM_004461	NP_004452
	mt	*BC009115*	NM_138395	NP_612404		cyt β	*PheHB*	NM_005687	NP_005678
						mt	*FARS1*	NM_006567	NP_006558
TrpRS	cyt	*WARS*	NM_004184	NP_004175	***ProRS***	cyt	*EPRS*	NM_004446	NP_004437
	mt	*WARS2*	NM_015836	NP_056651		***mt***		NM_152268	NP_689481
TyrRS	cyt	*YARS*	NM_003680	NP_003671	**SerRS**	cyt	*SARS*	NM_006513	NP_006504
	mt	*FLJ13995*	AK024057	BAB14806		**mt**	*SARSM*	NM_017827	NP_060297
ValRS	cyt	*VARS2*	NM_006295	NP_006286	***ThrRS***	cyt	*TARS*	NM_003191	NP_689508
	mt	*FLJ20504*	AK000511			***mt***		BC000541	AAH00541

[a] Cytosolic (cyt) and mitochondrial (mt) aaRSs are sorted alphabetically according to their belonging to class I (left part) or class II (right part) (*4*, *5*). Newly "in silico" identified aaRSs are in boldfaced italic font. Only the gene for GlnRS was not found. Accession numbers (acc. no.) are compatible with NCBI databases.

human mt-tRNAs (e.g., *18*−*20*, *23*). Aminoacylation properties of both enzymes were tested under the same conditions except that aspartylation was measured at 25 °C and tyrosylation at 37 °C. The nonphysiological lower temperature used for aspartylation was required to stabilize the structure of the in vitro transcribed substrate. The two recombinant enzymes are catalytically active, and both recognize and charge specific *E. coli* tRNAs with similar efficiencies (estimated by the ratio k_{cat}/K_M). Both enzymes aminoacylate also their cognate substrates produced by in vitro transcription, albeit with a 10- to 40-fold reduced efficiency than they charge *E. coli* native tRNAs.

Databank Mining for Completion of the Human Mitochondrial Aminoacyl-tRNA Synthetases Set. Cyberscreening within databases for the nine missing human mt-aaRS genes was done similarly to the search of human mt-AspRS and mt-TyrRS. Already known aaRS sequences of a given specificity were used as queries, and a search for a mt-targeting sequence was performed. Candidate sequences were found for eight mt-aaRSs, that is, AlaRS, ArgRS, AsnRS, CysRS, GluRS, ProRS, ThrRS, and ValRS. As anticipated, no gene for mt-GlnRS was found. The newly identified enzymes contain either of the consensus sequences defining the catalytic site of a synthetase. However, two particular cases have to be emphasized. Mt-ArgRS has an "-MKTR" motif instead of "KMSKS", a situation already reported for other ArgRSs (*38*), and human mt-AlaRS is deprived of motifs 1 and 2, also a known situation for AlaRSs from other organisms (*39*).

Tables 2 and 3 summarize the data collected for the new potential genes, as well as for the mt-AspRS and mt-TyrRS genes and those for the other already known human mt-aaRSs. Enzymes are sorted according to the synthetase classification, with five new specificities in each class (in boldface italic font). Table 2 collects the accession numbers, and Table 3 gives information on the genes (chromosomic location, number of exons, total length of the gene) and on the corresponding proteins (mt-targeting probability, size of the full-length protein, predicted size of the mature enzyme, sequence identity with related human cyt-aaRS).

Each of the eight new candidate sequences shows a high mt-targeting probability as obtained by predictive programs. Interestingly, the two sets of cyt- and mt-aaRS genes are clearly distinct since they have different loci, either on different chromosomes (ArgRSs, CysRSs, GluRSs, AlaRSs, AsnRSs, and ThrRSs) or within a same chromosome (ValRSs and ProRSs). As far as sequences are concerned, mt-aaRSs show only limited identity with their cytosolic counterparts, except for the known cases of GlyRSs (*12*, *13*) and LysRSs (*19*) for which it has been demonstrated that the cytosolic and mitochondrial enzymes originate from a single gene. Notice the particular high score of sequence identity of cyt- and mt-HisRSs, which has been explained by an inverted gene duplication (*14*, *15*).

DISCUSSION

Human Mt-AspRS and Mt-TyrRS Are of Eubacterial Type. Exploration of human databases revealed potential sequences for 10 mt-aaRS genes (i.e., AlaRS, ArgRS, AsnRS, AspRS, CysRS, GluRS, ProRS, ThrRS, TyrRS, and ValRS), leading thus toward the full set of mt-aaRSs. The sole exception concerns a gene for GlnRS. The new assignments are supported by (i) multiple sequences alignments with aaRS of same specificity from other organisms, (ii) the existence of typical catalytic site signature motifs, (iii) the prediction of an N-terminal mt-targeting sequence, (iv) the clear distinction between the sequences of these mt-aaRSs and their corresponding cytosolic counterparts, and (v) in the cases of mt-AspRS and mt-TyrRS, the functionality of the overexpressed and purified proteins.

Both mt-AspRS and mt-TyrRS are active enzymes, able to specifically aminoacylate *E. coli* native $tRNA^{Asp}$ and $tRNA^{Tyr}$, as well as in vitro transcribed human mt-tRNAs.

Table 3: Characteristics of Human Mitochondrial Aminoacyl-tRNA Synthetases[a]

	mt-targeting probability			genomic features			size (amino acids)		sequence comparison		
aaRSs	P	MP	cleavage site	X	no. of exons	total length (nt)	full length	mature	ident %	sim %	gap %
					Class I aaRSs						
ArgRS	0.997	0.781	after 16	VI (V)	20	75 618	578	562 (660)	28	46	8
CysRS	0.589	0.483	after 62	XIII (XI)	15	64 704	564	502 (748)	32	43	22
GlnRS				(III)				(775)			
GluRS	0.942	0.994	after 25	XVI (I)	9	32 980	523	498 (1440)	23	42	9
IleRS	0.774*	0.685	after 61	I (IX)	23	53 358	993	933 (1262)	28	44	12
LeuRS	0.972	0.783	**39 aa**	III (V)	20	153 073	903	**864** (1176)		not significant	
MetRS	0.992	0.982	**18 aa**	II (XII)	1	1 779	593	**575** (900)	24	39	10
TrpRS	0.995	0.735	**18 aa**	I (XIV)	6	109 446	360	**342** (471)		not significant	
TyrRS	0.944	0.974	after 16	XII (I)	5	8 630	477	461 (528)		not significant	
ValRS	0.994	0.828	after 29	VI (VI)	30	11 364	993	964 (1264)	45	58	9
					Class II aaRSs						
AlaRS	0.973	0.998	after 68	VI (XVI)	22	13 672	985	917 (968)	45	62	3
AsnRS	0.984	0.974	after 14	XI (XVIII)	14	137 812	477	463 (548)	29	44	9
AspRS	0.816	0.998	after 47	I (II)	17	32 475	645	597 (500)	23	36	26
GlyRS	0.999	0.999	**54 aa**	VII (VII)	17	39 005	739	**685** (685)	100		0
HisRS	0.987	0.972	after 34	V (V)	13	6 900	506	472 (509)	76	87	0
LysRS	0.784	0.973	**16 aa**	XVI (XVI)	15	16 530	625	**609** (597)	98	98	0
PheRS	0.951	0.974	**37 aa**	VI (α-XIX/β-II)	6	403 026	451	**414** (α-508/β-589)	not significant (α- or β- chains)		
ProRS	0.786*	0.986	after 47	I (I)	1	1 428	475	428 (1440)		not significant	
SerRS	0.984	0.986	after 20	XIX (I)	16	15 127	518	498 (514)	28	49	5
ThrRS	0.991	0.951	after 39	I (V)	18	19 600	718	679 (712)	58	69	2

[a] AaRSs are sorted according to their belonging to class I (upper part) or class II (lower part) (*4*, *5*). Mitochondrial-targeting probability was evaluated using the predictive programs Predotar (P) (or TargetP when indicated by ∗) or MitoProt (MP) (for Web sites see Materials and Methods). Mt-targeting sequence cleavage sites are either predicted by the above-mentioned programs (indicated "after xx") or based on biochemical experiments (boldfaced characters). Genomic features of human mt-aaRSs include chromosomic location (X) in roman characters (those for cyt-aaRSs are recalled in parentheses), number (no.) of exons, and total gene size. Chromosomic location has been established by standard Blastn of the coding sequences against the human chromosome database, leading to hits representative of the exons. Protein sizes correspond either to full-length proteins (including the mt-targeting sequence) or to mature enzymes (in bold when experimental data are available). Protein sizes of cyt-aaRSs are recalled in parentheses. Comparison with human cyt-aaRSs indicates percentages of identity (ident), similarity (sim), and gap as calculated using standard pairwise Blast. For cyt-PheRS (composed of two subunits), comparisons have been performed with both chains. Mt-ProRS has been compared with the bifunctional cyt-GluProRS.

Catalytic activities obtained with transcripts are however less efficient (10- to 40-fold) than those obtained with native *E. coli* molecules, a situation already reported for other human mitochondrial aminoacylation systems (*18*, *20*, *23*, *36*, *40*, *41*). This might be accounted for either (i) by a foreseen higher structural flexibility of mt-tRNAs due to their biased nucleotide composition (75% and 57% of A/U residues in mt-tRNA^{Asp} and mt-tRNA^{Tyr}, respectively, ref *42*) or (ii) by the absence within in vitro transcripts of posttranscriptional modifications, known to have a stabilization effect on RNA folding (*43*) and in particular on human mt-tRNA folding (e.g., *44*, *45*). When tested with native *E. coli* substrates, efficiencies of the two enzymes are in a range comparable to those observed for the corresponding cognate *E. coli* enzymes tested with their native tRNA. While data are not strictly comparable due to variations in aminoacylation conditions, the efficiency of human mt-TyRS is very close to that of *E. coli* TyrRS (315×10^{-3} $s^{-1} \cdot \mu M^{-1}$, ref *46*), and the efficiency of mt-AspRS is ∼20-fold lower than that of the *E. coli* enzyme (*47*). That some mammalian mt-aaRSs have a slower enzymatic activity than bacterial synthetases has already been documented (e.g., *18*, *20*). Interestingly, the *E. coli* and human mt-tRNAs are significantly different at the structural and primary sequence levels (Figure 4), which opens a number of questions as to their recognition by mt-aaRSs. Initial answers are available for both tyrosine (*48*) and aspartate systems (Fender et al., manuscript in preparation).

Sequence comparisons with aaRSs of same specificity show that both human mt-AspRS and mt-TyrRS fit into the expected modular organization of these families of enzymes (*24*, *25*). The N-terminal region of mt-AspRS (Figure 5A) corresponds to the anticodon-binding domain (aa 48−154) and its C-terminal part to the catalytic domain (aa 177−606), both connected via a hinge region. Interestingly, the mitochondrial enzyme possesses the typical eubacterial insertion (aa 328−456) and C-terminal extension (aa 607−645) domains and is thus distinguished from eukaryal and archæal AspRSs. Detailed sequence comparison within conserved domains pinpoints additional elements highlighting eubacterial features of mt-AspRS. For instance, residues T219 (corresponding to T169 in *E. coli*, located in the so-called flipping loop that closes their active sites when aspartate is bound) and Q280 (corresponding to Q231 in *E. coli*, in motif 2) are conserved solely in eubacterial AspRSs and are replaced by two serines in AspRSs from other phylae.

The modular organization of human mt-TyrRS is similar to that of other TyrRSs (Figure 5B) with an N-terminal catalytic domain (aa 32−296) followed by the C-terminal anticodon-binding region (aa 296−477). The catalytic domain presents the expected insertion domain, the so-called connective peptide CP1, here involved in dimerization and cross-species tRNA discrimination (*49*, *50*). It also appears that the anticodon-binding region is comparable to that of eubacterial TyrRSs with similar α-helical and C-terminal domains. However, on the basis of sequence comparisons, two subfamilies (represented, e.g., by *E. coli* and *T. thermophilus* TyrRSs, respectively) of eubacterial TyrRSs emerged (*51*). Human mt-TyrRS shares significantly higher sequence identity with the *E. coli*-type than *T. thermophilus*-type of eubacterial TyrRSs (see Figure 2). This α-helical/C-terminal organization clearly distinguishes from archæal enzymes (very short and low in sequence identity at the C-terminus) and from eukaryotic TyrRSs, especially the human cytosolic

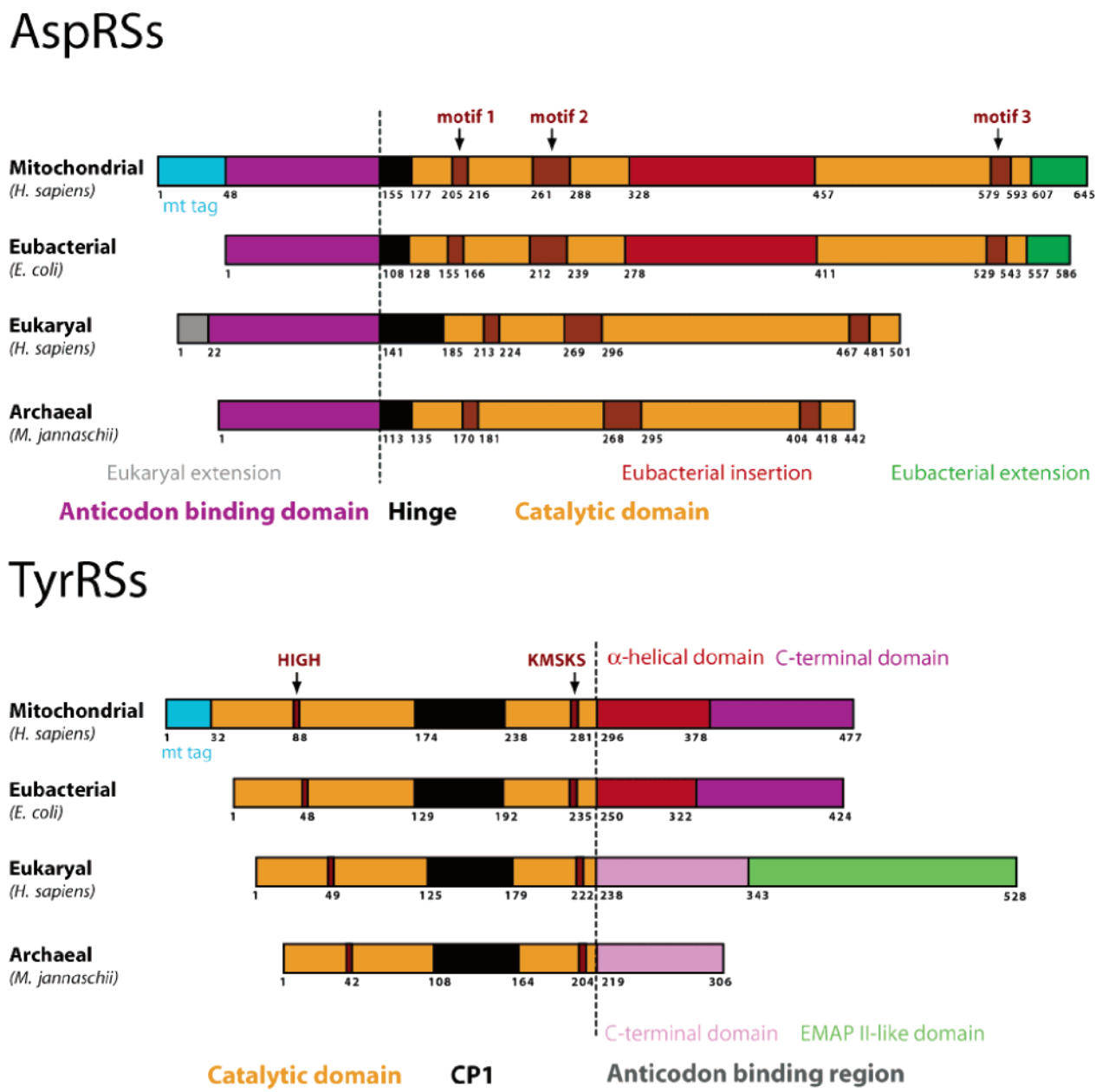

FIGURE 5: Modular organization of human mt-AspRS and mt-TyrRS compared with those from bacteria, eukarya, and archæa. The aaRSs are lined up at the junction between their anticodon-binding and catalytic domains (dotted lines). Amino acid numbering corresponds to that of the aaRSs from organisms given in parentheses. Each structural module is differently colored.

enzyme, which possesses a long EMAP II-like domain (*32*). This low degree in conservation for C-terminal modules is a peculiarity of TyrRSs (e.g., *25*). Regarding N-terminal domains, human cyt-TyrRS presents an interleukin 8-like cytokine activity related to an ELR signature motif within the Rossmann-fold domain (*52*), a motif absent within mt-TyrRS (ELR is replaced by REA). Altogether, these differences (absence of EMAP II-like domain and ELR motif) suggest that no cytokine activities might be anticipated for the mitochondrial enzyme neither in its C- nor N-terminus domain. In summary, both mt-AspRS and mt-TyrRS are of eubacterial type. However, in the case of mt-TyrRS, fine inspection of the sequence of the catalytic domain shows some archæal features, supported by functional data (*48*).

The eubacterial character of mt-AspRS and mt-TyrRS is in line with an endosymbiotic origin of mitochondria, believed to be a remnant eubacteria engulfed by primitive eukaryotes (*53*, *54*). In such a scenario, the present day organelle genome would result from the progressive transfer of endosymbiont aaRS genes to the nuclear genome concomitantly with the loss of other genes. Alternatively, mt-aaRS genes could also originate from duplication of a cyt-aaRS gene (e.g., *39*, *55*, *56*). The low sequence identity of human mt-AspRS and mt-TyrRS with the corresponding cyt-aaRSs, combined with their high sequence identities with eubacterial aaRSs and a modular organization closer to eubacterial aaRSs, favors the gene transfer hypothesis from the mitochondrial progenitor to the nucleus for these two enzymes.

Mitochondrial and Cytosolic Human Aminoacyl-tRNA Synthetases Are Encoded by Two Sets of Genes. Prediction of gene/protein sequences for eight additional mt-aaRSs allows now for a global view on the full set of these enzymes in human. Reconstruction of all mt-aaRS genomic sequences (established by standard Blastn of coding sequences against the human chromosome database; see Table 3) gives an

insight into their gene organization. The total length occupied by the different genes ranges from 1428 nt (ProRS) to 403 026 nt (PheRS), a variability as large as the number of exons ranking from 1 (ProRS) to 30 (ValRS). At the protein level, mature mt-aaRSs (deprived of a the mt-tag) contain (or are predicted to contain) 360−993 aa, making them 3−36% smaller than the corresponding cytosolic enzymes. As a noticeable exception, mt-AspRS is larger (596 aa) than cyt-AspRS (500 aa) due to the large eubacterial insertion domain. Interestingly, two independent coding sequences have been found for mt-GluRS and mt-ProRS, at opposite ends on a single gene for both activities in the human cytosol leading to a bifunctional GluProRS (*57*). Cyt-GluProRS (1440 aa) is larger than the combination of mt-GluRS (497 aa) and mt-ProRS (427 aa). Another particular situation concerns PheRSs, with the cyt-aaRS being tetrameric ($\alpha_2\beta_2$), while the mt-aaRS is monomeric (α) (*18*). Both subunits (508 and 589 aa) of the cytosolic enzyme are larger than the single subunit of the mitochondrial enzyme (414 aa). The drastic change in mt- and cyt-PheRSs quaternary structures was also reported in yeast (*58*).

The striking outcome of the completed gene identification is that mitochondrial synthetases are encoded by genes different from those coding for the cyt-aaRS of same specificity. Sole exceptions concern the already reported cases of GlyRSs (*12*, *13*) and LysRSs (*19*). This gene distribution is different from the situation in organisms for which sufficient information is available (reviewed, e.g., in ref *10*). For example, in *S. cerevisiae* and in *Arabidopsis thaliana*, a larger number of cytosolic and mitochondrial synthetases are generated from a single gene. This is the case for yeast HisRSs (*59*) and ValRSs (*60*) and *A. thaliana* AlaRSs (*61*), ThrRSs, and ValRSs (*62*).

Perspectives. The identification/annotation step of the full set of human mt-aaRS genes opens also a large number of research lines at fundamental and at forensic levels. Indeed, more neurodegenerative disorders are correlated to point mutations in human mt-tRNA genes (e.g., *63*−*66*), and defects in aminoacylation properties of these mutated tRNAs are likely targets for molecular impacts (*42*). Immediate access to active mt-AspRS and mt-TyrRS allows more biochemical investigation of these enzymes, including their relationships with cognate tRNAs and exploration of their aminoacylation identity elements. Experiments toward this aim are underway.

The case of mt-GlnRS remains puzzling. We were unable to detect a candidate gene for this enzyme. Among possible explanations, it can be proposed either that the sequence of human mt-GlnRS has evolved so much that it has become unrelated to any of the known GlnRSs from other organisms or that its function is fulfilled by a mt-addressed version of the cyt-GlnRS. So far, we were unable to detect sequences that may unravel these two hypotheses. Another alternative, in line with previous reports suggesting that this enzyme does not exist (*67*, *68*), would be that synthesis of mt-Gln-tRNA^{Gln} occurs via an indirect pathway involving two steps, misacylation of tRNA^{Gln} by GluRS yielding Glu-tRNA^{Gln} followed by Glu-amidation (*69*). This hypothesis is supported by the existence of a gene homologuous to glutaminyl-amidotransferases in the human genome (accession number NP_004555). However, further investigations are needed to decipher the glutaminylation pathway in human mitochondria.

From an evolutionary point of view, the present work further confirms and extends the view that human mt- and cyt-aaRSs are coded by two different sets of genes. However, in humans, there are two exceptions as described above. In other organisms, this difference can be greater like in yeast and *A. thaliana.* An outcoming question is to know whether a situation could occur in nature where the two sets of genes are completely independent. Inspection of the newly sequenced genomes should shed light on this problem.

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