THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 23, pp. 15980–15988, June 9, 2006

Loss of a Primordial Identity Element for a Mammalian Mitochondrial Aminoacylation System*[S]

Received for publication, October 27, 2005, and in revised form, April 5, 2006 Published, JBC Papers in Press, April 5, 2006, DOI 10.1074/jbc.M511633200

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In mammalian mitochondria the translational machinery is of dual origin with tRNAs encoded by a simplified and rapidly evolving mitochondrial (mt) genome and aminoacyl-tRNA synthetases (aaRS) coded by the nuclear genome, and imported. Mt-tRNAs are atypical with biased sequences, size variations in loops and stems, and absence of residues forming classical tertiary interactions, whereas synthetases appear typical. This raises questions about identity elements in mt-tRNAs and adaptation of their cognate mt-aaRSs. We have explored here the human mt-aspartate system in which a prokaryotic-type AspRS, highly similar to the *Escherichia coli* enzyme, recognizes a bizarre tRNA^{Asp}. Analysis of human mt-tRNA^{Asp} transcripts confirms the identity role of the GUC anticodon as in other aspartylation systems but reveals the non-involvement of position 73. This position is otherwise known as the site of a universally conserved major aspartate identity element, G73, also known as a primordial identity signal. In mt-tRNA^{Asp}, position 73 can be occupied by any of the four nucleotides without affecting aspartylation. Sequence alignments of various AspRSs allowed placing Gly-269 at a position occupied by Asp-220, the residue contacting G73 in the crystallographic structure of *E. coli* AspRS-tRNA^{Asp} complex. Replacing this glycine by an aspartate renders human mt-AspRS more discriminative to G73. Restriction in the aspartylation identity set, driven by a rapid mutagenic rate of the mt-genome, suggests a reverse evolution of the mt-tRNA^{Asp} identity elements in regard to its bacterial ancestor.

Specific aminoacylation of transfer RNAs (tRNA) by their cognate aminoacyl-tRNA synthetases (aaRS)[4] is a key step in translation of genetic information. Rules governing recognition between partner macromolecules have been well studied for a large number of prokaryotic and cytosolic eukaryotic systems (1–3). They rely on identity nucleotides, mainly located at both extremities of the tRNA L-shape, that contact specific amino acids in the aaRS structure. Composed of major and minor elements, identity sets are usually conserved during evolution, as is the architecture of tRNA (1, 3). However, they can be completed by idiosyncratic subtleties such as cryptic (4) or permissive (5) elements and structural features including flexibility and the overall tRNA scaffold (6, 7). Despite that, identity sets remain mainly unknown for organellar tRNAs, in particular for mammalian mitochondrial (mt)-tRNAs, a group of atypical macromolecules.

Mammalian mt-tRNAs differ from classical tRNAs at the structural level and in regard to potential identity sets (8). Coded by the mt genome, they display non-canonical features and significantly degenerated sequences (8, 9). A majority (14 of 22) possess a biased nucleotide composition, unusual sizes of D- and/or T-loops, and miss otherwise conserved residues required for three-dimensional folding. Identity elements are only sparsely known. According to the endosymbiotic hypothesis on the origin of mitochondria (10) and the ability of mt-aaRSs to aminoacylate *Escherichia coli* tRNAs (11), identity elements of *E. coli* tRNAs were searched in the corresponding mammalian mt-tRNA sequences, and some have indeed been found (8). Conversely for the large sequence and structural degeneracy of mammalian mt-tRNAs, corresponding mt-aaRSs seem not to depart from the already known synthetases (Refs. 12, 13 and references therein). They are nuclear encoded and possess an mt-targeting signal. Recent completion of the full set of human (Hs) mt-aaRS genes confirms the view that, except for GlyRS[5] and LysRS, mt and cytosolic aaRSs are coded by two different sets of genes (12). The human synthetases of mt location have no apparent structural peculiarities compared with related synthetases (except PheRS, which is a monomer instead of an $\alpha_2\beta_2$ tetramer). They belong to the expected aaRS classes because they possess either of the two signatures in the catalytic domain (Rossmann-fold for class I; semi-conserved motifs 1, 2, and 3 for class II) (14, 15). In a larger frame, mt-aaRSs from various organisms present, with a few exceptions, typical and expected features of synthetases (13, 16, 17).

Apposition of "bizarre" tRNAs *versus* standard aaRSs raises a question as to the rules governing recognition and specific aminoacylation. To shed light on this question, we have explored the Hs mt aspartylation system. This choice is guided by the extensive knowledge of aspartylation identity elements in various organisms including *E. coli* (18, 19), *Saccharomyces cerevisiae* (20–23), and *Thermus thermophilus* (24). Further, Hs mt-AspRS was recently cloned and overexpressed and is one of the few mt synthetases with enough solubility to be analyzed biochemically (12).

Hs mt-tRNA^{Asp} presents characteristic mammalian mt-tRNA features. When compared with "classical" tRNAs (Fig. 1, *A* and *B*), it has shorter D- and T-loops and a biased nucleotide composition with 29 A, 7 G, 24 U, 11 C leading to a high content of weak base pairs (16 A-U and G-U pairs *versus* only 5 G-C pairs). Typical signals important for tertiary interactions are missing, such as residues G18 and G19 in the D-loop and the UUC sequence (the precursor of TΨC) in the T-loop involved in long-range interactions. Altogether, only 5 of the 9 expected tertiary

* This work was supported in part by Centre National de la Recherche Scientifique, Université Louis Pasteur Strasbourg, Association Française contre les Myopathies, and Action Concertée Incitative (ACI-BCMS 146). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

[1] Supported by a fellowship from Ministère de l'Enseignement Supérieur et de la Recherche.

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[4] The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; mt, mitochondrial; WT, wild type.

[5] aaRS stands for 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.

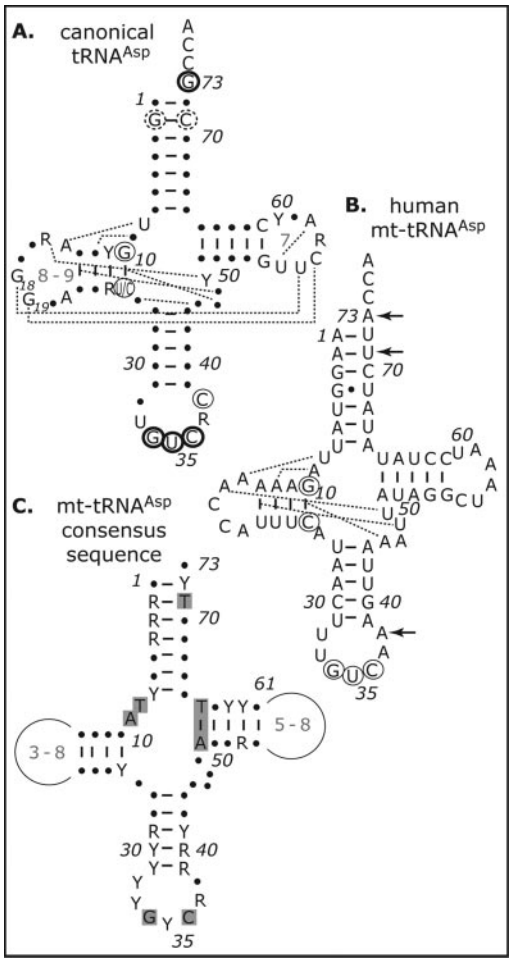

FIGURE 1. **Human mt-tRNA^{Asp} is structurally bizarre and misses some classical aspartate identity elements.** *A*, cloverleaf representation of a canonical tRNA with strictly conserved residues and tertiary interactions (*dotted lines*) emphasized. *Y* and *R* stand for pyrimidine and purine, respectively. Major (*thick circles*) and minor (*thin circles*) aspartate identity elements in yeast, *T. thermophilus*, and *E. coli* (26) are shown. The *E. coli* minor element is in *dotted circles*. Numbering is according to Ref. 41. *B*, cloverleaf representation of Hs mt-tRNA^{Asp} (59) with tentative tertiary interactions (*dotted lines*). Notice the particular D- and T-loops. Expected identity elements are *circled*, and missing elements pinpointed by *arrows*. *C*, consensus sequence of mt-tRNA^{Asp} genes from 136 mammals. The non-encoded CCA end is omitted.

interactions involved in an L-shaped three-dimensional folding (25) could be predicted. These structural features suggest a much more flexible structure for mt-tRNA^{Asp} than for classical tRNAs (8).

Sequence comparison of classical (*i.e.* non-mt) tRNA^{Asp} molecules highlighted conserved nucleotides that have proven to be major aspartate identity elements in all systems tested so far (reviewed in Refs. 26, 27). They correspond to the discriminator residue G73 and the anticodon triplet G34-U35-C36. Aspartate identity is further defined by additional elements of minor strength such as residue C38 and base pair G10-U/C25, and further in *E. coli* by the species-specific G2-C71 pair (Fig. 1*A*). Surprisingly, Hs mt-tRNA^{Asp} presents the potential major elements within the anticodon triplet, but not the discriminator base G73. Further, within the minor elements, only the pair G10-C25 is present whereas C38, as well as the minor *E. coli* signal G2-C71, is missing. Analysis of the consensus of 136 mammalian mt-tRNA^{Asp} sequences (Fig. 1*C*) confirms a strong conservation (albeit not total) of the anticodon triplet nucleotides and the absence of nucleotide conservation at position 73 and of the minor elements. It highlights base pairs R2-U71 (R for purine) and A49-U65 as strongly conserved, designating them as potential identity elements. This analysis leads to the hypothesis that Hs mt-tRNA^{Asp} presents either a reduced or an alternate identity set compared with the so far known other aspartate tRNAs.

In contrast, cognate nuclear-encoded Hs mt-AspRS, recently characterized (12), displays standard features and resembles known AspRSs. Markedly, it is a class II synthetase, has the typical bacterial insertion and C-terminal extension, and shares >40% of strictly identical residues with *E. coli* AspRS.

We have shown here that Hs mt-tRNA^{Asp} conserves GUC aspartate anticodon as identity elements but not the discriminator position 73. This indicates simplified aspartate identity rules in the Hs mt system. Sequence comparison shows a local structural deviation in the mt enzyme next to the discriminator position in the tRNA. Accordingly, an mt-AspRS could be engineered to partially restore its discriminating capacity for G73. This is in favor of a local and focused adaptation of the slowly evolving nuclear-encoded enzyme, allowing for the unimportance of residue 73 rather than for recognition of an alternative nucleotide at this position. These data will be discussed in light of molecular evolution of tRNA aminoacylation systems in the peculiar mt context.

EXPERIMENTAL PROCEDURES

Materials—Synthetic genes of yeast (28), *E. coli* (20), and Hs mt-tRNA^{Asp} (12) were previously cloned. The *T. thermophilus* tRNA^{Asp} transcript and *E. coli* AspRS were gifts from H. Becker and G. Eriani. Hs mt-tRNA^{Tyr} transcripts were gifts from L. Bonnefond and J. Rudinger-Thirion. Hs mt-AspRS was cloned (plasmid pQE70-mt-AspRS), overexpressed, and purified as described (12). T7 RNA polymerase was purified as described (24). Oligonucleotides were from Sigma Genosys, restriction enzymes (BamHI, HindIII, and BstNI) from New England Biolabs, T4 DNA ligase from Qbiogen, and L-[^{3}H]aspartic acid (1.37 TBq/mmol) from Amersham Biosciences.

tRNA Gene Mutagenesis and in Vitro Transcription—Hs mt-tRNA^{Asp} variant genes were obtained by hybridization of 9 overlapping oligonucleotides, ligation between BamHI and HindIII sites of plasmid pTFMa (28), and transformation into TG1 cells. Mutant genes were obtained with the QuikChange^{TM} site-directed mutagenesis kit (Stratagene). These genes contain a hammerhead ribozyme (29) and tRNA sequences downstream the T7 polymerase promoter. A BstNI site coincidental with the 3′-end of the tRNA sequences allows synthesis of tRNAs ending with the expected CCA sequence. Transcription was as described (29).

mt-AspRS Mutagenesis and Expression—Plasmid pQE70-mt-AspRS was converted into pQE70-mt-AspRS/G269D using the QuikChange^{TM} site-directed mutagenesis kit (Stratagene) and transformation of *E. coli* TOP10 strain. Mt-AspRS/G269D was expressed and purified on nickel affinity column (12). Protein concentration was determined from A_{280} using an extinction coefficient calculated with ProtParam from Expasy tools ($\varepsilon = 43540\ M^{-1}\cdot cm^{-1}$).

Aminoacylation—Assays were performed as described (12) except that they were conducted at 15 instead of 25 °C. Buffer conditions were 50 mM HEPES-KOH, pH 7.5, 25 mM KCl, 12 mM $MgCl_2$, 2.5 mM ATP, 0.2 mg/ml of bovine serum albumin, 1 mM spermine, 32 μM [^{3}H]aspartic acid (208 GBq/mmol), and adequate amounts of transcripts and aaRS. Assays were performed in 50-μl samples. Transcripts were renatured (60 °C for 90 s in water and slow cooling down to room temperature) before aminoacylation was performed at 15 °C. Maximal charging levels (plateaus) were determined for 400 nM transcript and 680 nM synthetase (wild-type (WT) or G269D mutant AspRS). Under such conditions, plateaus determine the percentage of active molecules in each tRNA preparation (30). Plateau values varying between 40 and 80% were obtained and taken into account for K_m calculations. Synthetase activity was measured under excess substrate (8 μM) and 50 nM enzyme. Kinetic

parameters k_{cat} and K_m were derived from Lineweaver-Burk plots obtained under adequate ranges of tRNA (40 nM-1 μM) and AspRS (50–200 nM). For tRNA^{Asp} mutant C36U, k_{cat}/K_m was determined according to Ref. 31. Experiments were at least tripled on separate preparations of transcripts. Values varied up to 40%.

RESULTS AND DISCUSSION

Preliminary Considerations—Aspartate identity elements have been searched for by evaluation of the aspartylation properties of *in vitro* transcribed Hs mt-tRNAs. This strategy is of particular interest for human mt-tRNAs because it allows preparation of sufficient amounts of RNA either of WT or mutated sequence and has already been successfully applied for several human mt-tRNAs despite a number of drawbacks (reviewed in Ref. 32). Post-transcriptional modifications, absent in transcripts, may stabilize tRNA structure and/or provide a recognition element for interacting partners such as aaRSs. However, in the case of Hs mt-tRNA^{Asp} we believe that the absence of such modifications does not significantly affect aminoacylation properties. Comparative structure probing of native tRNA (extracted in nano-quantities from HeLa cells) and transcripts leads to similar results (see below and supplemental data). Further, post-transcriptional modifications have never been reported so far as a positive aspartate determinant (for instance, the yeast WT tRNA^{Asp} transcript is only 10-fold less efficiently aspartylated than native tRNA) (33), and this conclusion likely holds true in the Hs mt system. Indeed, Hs WT-tRNA^{Asp} transcript is aspartylated with only a 11.5-fold lower efficiency than native *E. coli* tRNA^{Asp} (12).

TABLE 1
Kinetic parameters for tRNA aminoacylation by human mt-AspRS and mt-AspRS/G269D

In vitro aminoacylation assays have been performed at 15 °C under experimental conditions as described under "Experimental Procedures." ND, not determined.

TRNA	k_{cat}	K_m	k_{cat}/K_m	Loss
	10^{-3} s^{-1}	μM	10^{-3} $s^{-1}\cdot\mu M^{-1}$	
Wt mt-AspRS				
wt tRNA^{Asp}	5.3 ± 1.7	0.19 ± 0.08	27.9	1.0
G73	3.4 ± 1.1	0.36 ± 0.15	9.4	2.9
C73	3.0 ± 1.0	0.09 ± 0.04	33.3	0.8
U73	2.4 ± 0.1	0.11 ± 0.02	21.8	1.2
A34	5.3 ± 1.3	2.18 ± 0.47	2.4	11.5
C34	24.8 ± 8.2	20.52 ± 8.52	1.2	23.2
U34	12.3 ± 4.3	6.09 ± 2.40	2.0	13.9
A35	ND	ND	ND	ND
C35	ND	ND	ND	ND
G35	ND	ND	ND	ND
A36	ND	ND	ND	ND
G36	ND	ND	ND	ND
U36	ND	ND	0.13	215
Mutant mt-AspRS/G269D				
wt tRNA^{Asp}	0.6 ± 0.1	0.10 ± 0.07	6.0	1.0
G73	1.9 ± 0.1	0.26 ± 0.07	7.7	0.8
C73	0.6 ± 0.2	0.08 ± 0.07	7.5	0.8
U73	0.7 ± 0.2	0.23 ± 0.11	3.0	2.0

However, as reported in several instances, handling of Hs mt-tRNAs remains tricky, especially those tRNAs with a high A, U, and C content ("light tRNAs") that are thermodynamically weak (as is the case for tRNA^{Asp}) (9). Thus, aminoacylation assays have to be performed at low temperatures, a compromise for optimal enzymatic activity and tRNA stability (34, 35). In the present study, aminoacylation assays were performed at 15 °C to allow for reproducible results. Lowering assay temperature from 25 to 15 °C reduces the catalytic efficiency for aspartylation only ~3-fold (k_{cat}/K_m of 77 10^{-3} $s^{-1}\cdot\mu M^{-1}$ at 25 °C (Ref. 12) and 27 10^{-3} $s^{-1}\cdot\mu M^{-1}$ at 15 °C; see Table 1). These efficiencies remain in a range similar to those observed for other Hs mt aminoacylation systems (12, 34–40).

The Aspartate Identity Set Is Simplified in Human Mitochondria—Identity elements for aspartylation were searched by a mutational analysis. A first series of variants included single point mutations at nucleotide positions that correspond to major aspartate identity determinants in yeast, *E. coli*, and *T. thermophilus*, *i.e.* positions 34, 35, 36, and 73. Mutations at positions 35 and 36 strongly impair aminoacylation (Fig. 2*A*) and lead at least to a loss of catalytic efficiency (k_{cat}/K_m) of 215-fold

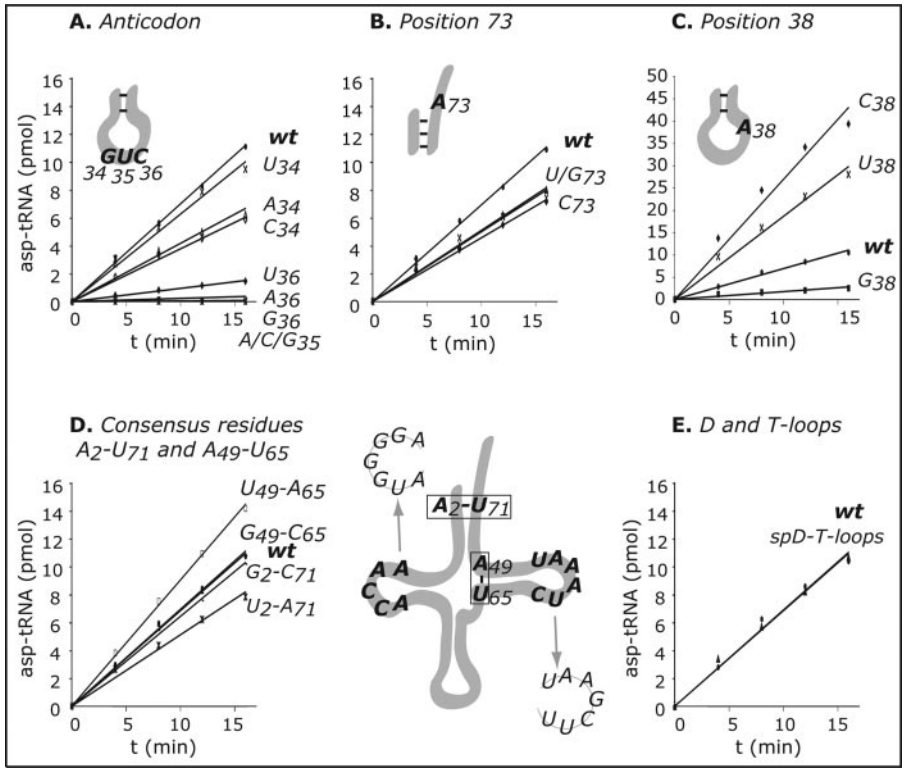

FIGURE 2. **Aspartylation of human mt-tRNA^{Asp} variants with human mt-AspRS.** Mutagenic analysis on variants at N34, 35, or 36 (*A*), discriminator N73 (*B*), and anticodon loop N38 (*C*). *Inserts* recall localization of the tested positions in the tRNA. *D*, variants with changes in the two conserved base pairs in mammalian mt-tRNA^{Asp} (*squared positions* on the schematized cloverleaf). *E*, structural variants of tRNA^{Asp} with D- and T-loops from *S. pombe* tRNA^{Asp} (see neighboring cloverleaf for loop sequences). Aminoacylation assays were performed as described under "Experimental Procedures" with 8 μM transcripts and 50 nM AspRS.

(Table 1). Mutations at position 34 moderately decrease aspartylation (Fig. 2*A*) with a loss in efficiency of 10- to 20-fold. In regard to the discriminator position 73, replacement of A73 by G73, C73, or U73 keeps significant aspartylation activities with rates close to those of WT (Fig. 2*B*) and kinetic parameters for the three variants very similar to those of WT (Table 1). Thus, although the anticodon nucleotides contribute to identity, position 73 is no longer an identity element in the human mitochondrial system.

Next, the contribution of nucleotides known as minor aspartate identity elements in bacterial or eukaryal systems was investigated. Replacement of A38 by G38 decreases aminoacylation whereas replacement by C38 or U38 increases it (Fig. 2*C*). This surprising stimulation (not observed for other aspartate tRNAs) could reveal either insertion of a positive identity element, suggesting that natural mt-tRNA sequence with A38 is not optimal for its cognate synthetase, or that a pyrimidine at position 38 leads to structural rearrangements within the anticodon domain favoring synthetase recognition. This hypothesis was tested by comparing solution structures of both WT mt-tRNA^{Asp} and variant C38. The two transcripts share essentially similar reactivities against enzymatic probes, indicative of a common global secondary structure. They differ, however, from native mt-tRNA^{Asp} at their D-domains (detailed data to be published elsewhere). Reactivity of nucleotides within the anticodon loop of the variants (supplemental Fig. S1) differs at subtle levels, those of variant C38 being midway between those in the post-transcriptionally modified native tRNA and those in the "naked" WT transcript. This suggests that the increased aminoacylation capacity of variant C38 is in part linked to a structural rearrangement of its anticodon loop. C38 may mimic the structural role of post-transcriptional modifications present in native tRNA.

Because of the importance of the anticodon loop in mt-tRNA^{Asp} aminoacylation, the contribution of position 37 to aspartylation was also tested, but none of the nucleotides introduced in place of A37 had an effect (results not shown). Thus, A37 is not an identity element.

Base pair G2-C71 is a minor identity element in *E. coli* tRNA^{Asp} (19). In Hs mt-tRNA^{Asp}, as in all other mammalian mt-tRNA^{Asp}, the sequence is conserved as R2-U71 (Fig. 1*C*), suggesting that it could be an important signal for mt-AspRS. Another strictly conserved signal is A49-U65 in the T-stem. Both base pairs were individually replaced by G-C or U-A (Fig. 2*D*). Aminoacylation properties of the four new variants are close to those of WT transcript, showing that corresponding WT nucleotides have no major contribution to mt aspartate identity.

An intriguing characteristic of mammalian mt-tRNAs is their unusual D- and T-loop ensembles with no classical size or obvious signals for tertiary interactions. The importance of these typical structural features was thus tested on aspartylation. Both loops of Hs mt-tRNA^{Asp} were replaced at once by those of *Schizosaccharomyces pombe* cytosolic tRNA^{Asp} (Fig. 2*E*), where classical tertiary interactions can take place. Aspartylation of this variant is not affected (Fig. 2*E*).

In summary, aspartylation of Hs mt-tRNA^{Asp} is restricted to the anticodon residues (U35 and C36 as strongest elements, G34 as moderate contributor). The discriminator residue 73 does not contribute. The primordial (22) and essential identity element G73 in bacterial and eukaryal aspartylation systems (26) is present in the three domains of life including organelles, with the sole exception of mitochondria from mammals (41). No additional identity element has been found (within the limit of the obvious candidates explored herein). It is of particular interest to notice that the mt aspartate identity set departs not only in a general manner from other aspartylation systems but also more specifically from the *E. coli* system despite the high sequence similarity of *E. coli* and Hs mt-AspRSs. Base pair 2–71 is not important and, within the anticodon triplet, the strength of the determinants is distinct: G34 = U35 > C36 in *E. coli* and G34 ≪ U35 = C36 in Hs mitochondria. Finally, the mt enzyme is not sensitive to structural features in the corner of the tRNA three-dimensional structure because it aminoacylates as well a Hs mt-tRNA^{Asp} transcript transplanted with canonical D- and T-loops like *E. coli* tRNA^{Asp} of classical structure (12).

Non-conservation of major identity elements in aminoacylation systems of a same specificity has been reported previously, especially non-conservation of the discriminator base in different organisms (3). For example, specific prolylation requires A73 and C1-G72 in *E. coli* whereas these positions are not involved in proline identity in Hs cytosol (42). U73 is a determinant for *E. coli* GlyRS whereas GlyRSs from archaea and eukaryotes recognize A73 (43). In these cases, variations in identity elements exist when comparing a bacterial system and either an archaeal or an eukaryal system, where synthetases are rather distinct. In the aspartate case, two synthetases of the same evolutionary origin obey different aminoacylation rules. *E. coli* AspRS and the bacterial type Hs mt-AspRS are very similar, but one requires strong identity elements at both ends of its substrate (the anticodon and the discriminator base) whereas the other relies only on the anticodon. A similar situation has been reported in *Arabidopsis thaliana* for a minor identity element with a prokaryotic-type mt-GlyRS not sensitive to residue 73, whereas *E. coli* enzyme is dependent on U73 (44).

Understanding Non-discrimination of Residue 73 for Recognition by mt-AspRS—The striking unimportance of residue 73 for Hs mt-AspRS, as opposed to its importance for bacterial or eukaryal AspRSs, has been tackled. Because of the very high similarity of the Hs mt enzyme with the *E. coli* AspRS, functional differences have been searched between these two enzymes. First, their cross-aminoacylation properties have been evaluated. Mt-AspRS was found to have a broad charging spectrum including, in addition to its cognate tRNA, tRNA^{Asp} from *E. coli*, *T. thermophilus*, and *S. cerevisiae* tRNA^{Asp} (supplemental Fig. S2*A*), which all possess the full set of aspartate identity elements and classical structural features. Of particular interest is the tRNA from *T. thermophilus,* which is especially GC rich and thus of opposite thermodynamic stability to the cognate mt-tRNA substrate. In contrast, *E. coli* AspRS aminoacylates tRNA^{Asp} from Hs cytoplasm (not shown) but not from yeast (45) nor does it aspartylate the Hs mt-tRNA^{Asp} (supplemental Fig. S2*B*). Rejection of the mt-tRNA by the *E. coli* enzyme may simply be due to the absence of the major identity element G73. However, *E. coli* AspRS does not recognize a variant mt-tRNA^{Asp} with G73 (and thus with the full set of major identity elements). The charging level is <1% (supplemental Fig. S2*B*). These data are in favor of (i) non-adapted structural scaffold of the mt-tRNA for the *E. coli* enzyme, (ii) and/or missing identity elements, (iii) and/or the presence of antideterminants in Hs mt-tRNA^{Asp} hindering productive aspartylation. In other words, two synthetases of the same evolutionary origin obey different aminoacylation rules.

Sequence comparison of mt- and *E. coli* AspRSs was then used to identify structural differences potentially responsible for non-discrimination of position 73 by mt-AspRS (Fig. 3*A*). Arginine (Arg-222) and aspartate (Asp-220) in motif 2 within the catalytic domain are conserved in all known bacterial AspRSs and establish specific contacts with G73. Whereas Arg-222 is conserved in mt-AspRS (Arg-271), Asp-220 is replaced by a glycine (Gly-269), designating this residue as a potential candidate contributing for non-discrimination of G73. Based on these observations, Gly-269 was replaced by aspartate 269, and aspartylation capacity of mt-AspRS/G269D was tested in the presence of WT mt-tRNA^{Asp} and the three variants of position 73 (C73, G73, and U73). Residues of *E. coli* AspRS specifically contacting G73 are displayed in

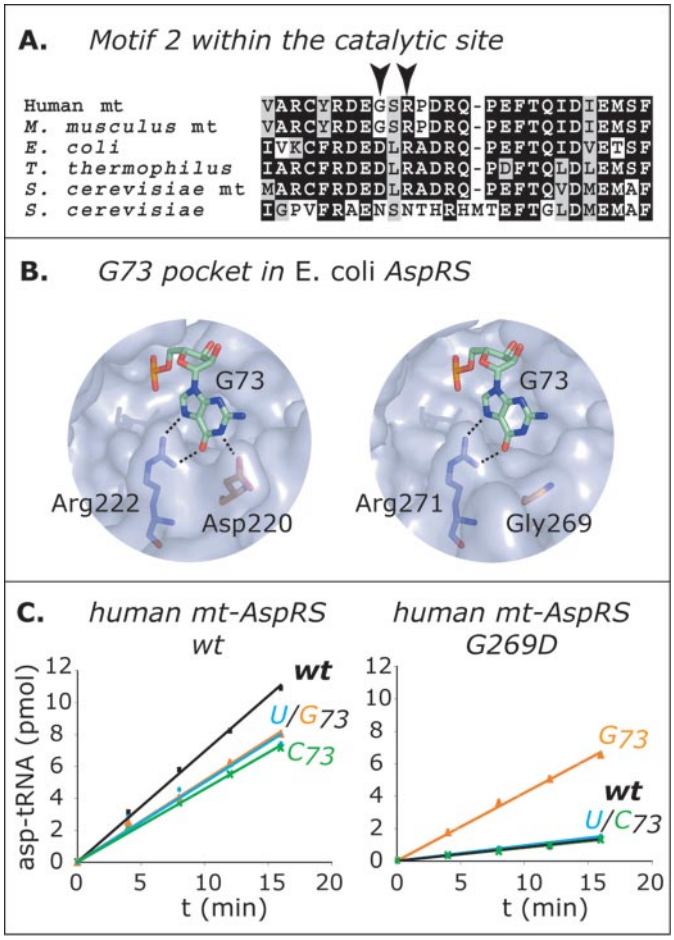

FIGURE 3. **Targeting relaxed specificity of human mt-AspRS.** *A*, sequence alignment of motif 2 of selected AspRSs (NCBI accession numbers: Hs mt, translated from AL833633; *M. musculus* mt, NP_76623; *E. coli*, P21889; *T. thermophilus*, P36419; *S. cerevisiae* mt, NP_015221; *S. cerevisiae*, P04802). *Black* and *gray boxes* show strictly identical and similar residues, respectively. *Arrowheads* locate *E. coli* residues Asp-220 and Arg-222 specifically contacting base G73 in the crystallographic structure of the complex (46). *B*, zooming into the binding pocket of residue G73 in the structure of *E. coli* complex (*left*, WT sequence, numbering according to *E. coli* AspRS; *right*, mutated sequence with replacement of Asp-269 into Gly-269, numbering according to mt-AspRS). *C*, aminoacylation by WT (*left*) and mutated (*right*) mt-AspRSs of *in vitro* transcribed WT and variant mt-tRNA^{Asp}. Reactions were performed under saturating tRNA concentrations (8 μM transcripts, 50 nM enzyme).

Fig. 3*B* on the crystallographic structure of the *E. coli* AspRS-tRNA^{Asp} complex (46). Activity of mt-AspRS/G269D, as estimated by maximal rate measurements, is ~10-fold decreased for WT mt-tRNA^{Asp} compared with the wild-type enzyme. In contrast to WT mt-AspRS, which recognizes quite similarly the four substrates, mt-AspRS/G269D is more sensitive to variant G73 (Fig. 3*C*). A detailed kinetic analysis likewise yielded a k_{cat} value of the G73 variant ~3-fold increased compared with WT mt-tRNA^{Asp} but showed also a K_m increase (Table 1) leading to an overall comparable aminoacylation efficiency of WT and variant G73 by mt-AspRS/G269D. The presence of Gly-269 in WT mt-AspRS likely reduces the number of direct contacts (H-bonds) with base 73 and thus may relax stringency within the protein region involved in mt-tRNA^{Asp} 3′-end recognition. However, the moderate kinetic effects observed for the single mutation on residue 269 suggest that other structural features, still to be discovered, contribute as well to the unimportance of residue 73 in aspartylation identity of human mt-tRNA^{Asp}. In support of this view we note that specificity alterations or recognition switches reported for non-mt aaRSs required mutation of several amino acids close to tRNA identity residues, either in the anticodon (*e.g.* Refs. 47–49) or, like here, in the accepting stem (*e.g.* Refs. 50, 51).

Mt-AspRS Adaptation to mt-tRNA Rapid Evolution—Mitochondria originate from engulfment of bacteria by primitive eukaryotes with progressive transfer of endosymbiont genes to the nuclear genome and concomitant loss of other genes (10, 52). As a result, mammalian mt genomes were reduced to a set of genes coding for only 13 proteins, 2 rRNAs, and 22 tRNAs. These genomes are subjected to a high mutational rate because of the combination of oxidative environment and limited DNA repair activity. Accordingly, mammalian mt-tRNA acquired a characteristic sequence degeneracy (53, 54) leading to strong polymorphism (55) and pathology-related mutations (56–58). In addition, the biased nucleotide composition of the mt genome (59) (high A/U/C content) favors what could be called a structural polymorphism, *i.e.* less stable two-dimensional and more flexible three-dimensional structures of mt-tRNAs compared with classical tRNAs.

Mammalian mt-aaRSs are nuclear encoded and sustain a low mutational rate. Their genes originate either from a transfer from the original endosymbiont or from duplication of the initial nuclear gene coding for the cytosolic synthetase of the host (13). A limited number of mutations along the tRNA-interacting surface are probably sufficient to enable mt-AspRS to be more tolerant of sequence and conformation variations than the *E. coli* enzyme. This is first illustrated by the capacity of mt-AspRS to charge a broad population of heterologous tRNAs, whereas the *E. coli* ortholog cannot recognize mt-tRNAs. Second, the low sensitivity to the discriminator base is an indication that the tRNA identity rules have been relaxed. In conclusion, evolution of the aspartate system in the mt context has consisted of a reduction in the number of identity elements and extension in tolerance to structural fluctuations.

Why a Restricted Identity Set Can Be Sufficient in Mitochondria—The mt translational machinery synthesizes only 13 proteins, all subunits of the respiratory chain complexes and thus involved in energy synthesis. Restriction of the aspartate aminoacylation identity set to the anticodon loop raises questions about its sufficiency to guarantee specific aminoacylation in the organelle. There are only 22 tRNAs in mammalian mitochondria; some share one or two nucleotides with tRNA^{Asp} anticodon triplet (tRNA^{Glu} with the strongest aspartate elements U35 and C36; tRNA^{His}, tRNA^{Asn}, tRNA^{Tyr} with G34 and U35; and tRNA^{Lys}, tRNA^{Val}, and tRNA^{Gln} with U35, C36, and U35) and would thus be candidates for mischarging by AspRS. However, attempts to aminoacylate *in vitro* transcribed Hs mt-tRNA^{Tyr} were not successful (not shown), although this tRNA is fully aminoacylated by its cognate mt-TyrRS. Further, a variant mt-tRNA^{Tyr} containing a complete GUC aspartate anticodon triplet does not become a substrate for AspRS. This restricted mischarging of mt-tRNAs by AspRS strongly suggests the presence of antideterminants within these tRNAs and/or limitations in their conformational adaptation required for non-cognate catalysis.

Only limited data are available on tRNA identity in mammalian mitochondria (32). However, it has been postulated that mt-tRNAs contain fewer sequence-specific recognition elements in addition to their anticodons because individual mt-tRNAs are so distinguishable that intricate recognition mechanisms seem unnecessary (11). This view is now experimentally validated for a few systems. In addition to the aspartate system, Hs bacterial-type mt-TyrRS does not discriminate base pair 1–72 although it is a strong identity element for *E. coli* TyrRS (60), and bovine mt-SerRS does not require the variable domain but rather the T-loop (especially A58) and nucleotides replacing the D-loop (61, 62). Such restricted identity sets in mt-tRNAs may be correlated with the lower catalytic efficiency of mt aminoacylation systems compared with bacterial or eukaryal cytosolic systems (9).

Accuracy of mitochondrial protein synthesis remains an open question. In a systematic investigation of bovine mt-tRNAs, it was shown

that mt-SerRS markedly misaminoacylates several of them (63). Several reports bring strong support to the fact that, contrary to other synthetases, mt-ProRS (64), mt-LeuRS (65), and mt-PheRS (66) have lost their editing activity, *i.e.* the proofreading step for errors in amino acid selection during the aminoacylation process. Interestingly, mt-LeuRS ensures fidelity thanks to a higher specificity for its cognate amino acid restricting the error rate of mischarging (65). In the case of mt-PheRS, it remains open whether quality control in mitochondria is focused on another step than editing (for example, via a more efficient protein degradation machinery) or whether translation in mitochondria is inherently less accurate (66).

Evolution and Mitochondria—Evolution of aminoacylation systems has been discussed in several instances (67–69). Primordial systems were likely composed of minihelices ("ur" acceptor stems) recognized by aaRS ancestors restricted to their catalytic core (70). An early "operational RNA code" typically formed of the three first tRNA acceptor stem base pairs and of residue N73, fairly named "discriminator base", may have preceded emergence of the genetic code (71). Subsequent evolution of aminoacylation systems was driven by insertion of additional tRNA domains and aaRS modules with concomitant *expansion* of tRNA identity sets, now composed of conserved major elements and idiosyncratic species-specific minor features. The present study on an mt aspartylation system reveals loss of a strongly conserved major identity element, G73. This *restriction* suggests a reverse evolution of this aminoacylation system in regard to its bacterial-type ancestor. Residue G73 was likely the primordial aspartate identity element because specific aminoacylation of minimalist tRNA substrates is possible (22). Its loss as a major aspartate element from bacteria to bacteria-like (Hs mt) systems illustrates a rupture of the operational RNA code within the aforementioned evolutionary-related biological systems. This situation was likely driven by the high mutational rate of the mt genome but also by a less stringent need of intricate identity rules due to a simplified tRNA world in mitochondria. The synthetase has adapted to allow for preservation of complementarity with its substrate by mutation of at least a critical aspartate residue into a glycine. One may question whether, because of the high tRNA degeneracy, reverse evolution of mt aminoacylation systems is a general trend in mammals?

Acknowledgments—J. Rudinger-Thirion, L. Bonnefond, and M. Frugier are acknowledged for helpful discussions, A. Clénet for technical assistance, and H. Becker, L. Bonnefond, G. Eriani, D. Kern, and J. Rudinger-Thirion for gifts of material.

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