Functional Divergence of a Unique C-terminal Domain of Leucyl-tRNA Synthetase to Accommodate Its Splicing and Aminoacylation Roles*  

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Leucyl-tRNA synthetase (LeuRS) performs dual essential roles in group I intron RNA splicing as well as protein synthesis within the yeast mitochondria. Deletions of the C terminus differentially impact the two functions of the enzyme in splicing and aminoacylation in vivo. Herein, we determined that a five-amino acid C-terminal deletion of LeuRS, which does not complement a null strain, can form a ternary complex with the b14 intron and its maturase splicing partner. However, the complex fails to stimulate splicing activity. The x-ray co-crystal structure of LeuRS showed that a C-terminal extension of about 60 amino acids forms a discrete domain, which is unique among the LeuRSs and interacts with the corner of the L-shaped tRNALeu. Interestingly, deletion of the entire yeast mitochondrial LeuRS C-terminal domain enhanced its aminoacylation and amino acid editing activities. In striking contrast, deletion of the corresponding C-terminal domain of Escherichia coli LeuRS abolished aminoacylation of tRNALeu and also amino acid editing of mischarged tRNA molecules. These results suggest that the role of the leucine-specific C-terminal domain in tRNA recognition for aminoacylation and amino acid editing has adapted differentially and with surprisingly opposite effects. We propose that the secondary role of yeast mitochondrial LeuRS in RNA splicing has impacted the functional evolution of this critical C-terminal domain.

Leucyl-tRNA synthetase is one of 20 aminoacyl-tRNA synthetases that are responsible for linking tRNA with its correct amino acid during protein synthesis (1). In the yeast mitochondrion, it is also critical for enabling splicing of the b14 and a14 introns from the cob and cox1α genes, respectively (2–4). LeuRS forms a ternary complex with the b14 matrase to confer splicing activity to the group I intron (5). LeuRSs from other organisms effectively substitute for the yeast mitochondrial LeuRS in null strains, suggesting that its role in splicing is aided by ancient adaptations of LeuRS that are universally conserved (6).

LeuRSs are class IA tRNA synthetases (7, 8) that contain a catalytic core composed of a Rossmann nucleotide-binding fold (9). Similar to other synthetases, its N-terminal canonical core is appended to a second C-terminal domain that is less conserved (10, 11). However, unlike other aminoacyl-tRNA synthetases, the C-terminal domain of LeuRS does not interact with the anticodon for recognition of its cognate tRNA (12, 13). Rather, a C-terminal extension of about 60 amino acids folds into a unique domain that is tethered via a flexible linker and interacts with the corner of the L-shaped tRNA (14). Specifically, the C-terminal domain of Thermus thermophilus LeuRS makes significant contacts with the G19:C56 tertiary base pair of the tRNA (14). In the absence of tRNALeu, this small unique C-terminal domain was disordered in the LeuRS apo-crystal structure (15). Deletion of the C-terminal domain has been shown to decrease aminoacylation activity of LeuRS (14, 16, 17). Mutational analysis has also demonstrated that the C terminus is required for LeuRS splicing activity (6, 18).

The binding of synthetases to their cognate tRNAs depends on identity elements found within the tRNA (19). For tRNALeu, the unpaired base at A73 appears to be the primary identity element required for base-specific recognition by LeuRS (12, 13, 20, 21). In addition, recognition by LeuRS also depends on the tRNA tertiary structure, particularly in the D-loop region (12, 22). For example, the triple interaction between A15:U48–A20a, and the invariants G18 and G19 are important for recognition of tRNALeu (13, 23).

We hypothesized that this C-terminal domain of LeuRS may be important for RNA binding and recognition and constructed deletion mutants for yeast mitochondrial and Escherichia coli LeuRS. Complementation experiments show that the C terminus is critical to the LeuRS splicing activity (18). Our results suggest that this unique domain of LeuRS has adapted differentially for aminoacylation. We propose that the role of yeast mitochondrial LeuRS in splicing impacts the adaptation of the C terminus to optimally accommodate both its splicing and aminoacylation roles.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotide primers were synthesized by MWG Biotech (High Point, NC). Tritium-labeled amino acids were acquired from Amersham Biosciences. T4 DNA ligase was
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purchased from Promega (Madison, WI). DpnI was obtained from New England Biolabs (Beverly, MA). Cloned Pfu DNA polymerase and dNTP mix were from Stratagene (La Jolla, CA). His-Select™ Resin from Sigma was used for protein purification.

RNA-dependent Two-hybrid Assays—Yeast two-hybrid cells were grown on solid agarose synthetic medium (Ura−, His−, Trp−) containing 2% galactose, 1% raffinose, and 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal). β-Galactosidase assays and reverse transcription-PCR analysis were carried out as described previously (5).

Protein Mutagenesis and Purification—A BamHI restriction site was introduced into the plasmid pYM3–17 (5) encoding the wild-type yeast mitochondrial LeuRS by site-directed PCR mutagenesis, using primers yMLRS–ΔC-Fwd (5’-GCC ACT ACT GAG GAC TAA GGA TCC GAC TCC ACG CTG GTC G-3’) and yMLRS–ΔC-Rev (5’-GAA ACT TTT TGA ATT TGT CGA CTT AGT CCT AAT CCA CCA TCG C-3’) by polymerase chain reaction-based mutagenesis. Likewise, for deletion mutagenesis of yeast mitochondrial LeuRS, a Sail restriction site was introduced into the plasmid pYM3–17 (5) encoding the wild-type yeast mitochondrial LeuRS by site-directed PCR mutagenesis, using primers yMLRS–ΔC-Fwd (5’-CCG ACT ACT GAG GAC TAA GGA TCC GAC TCC ACG CTG GTC G-3’) and yMLRS–ΔC-Rev (5’-GAA ACT TTT TGA ATT TGT CGA CTT AGT CCT AAT CCA CCA TCG C-3’). Each 50-μl PCR reaction contained 25 ng of plasmid DNA, 10 pmol of each forward and reverse primer, 0.2 mM dNTPs, 1 μl Pfu DNA polymerase buffer (Stratagene), and 2.5 units of Pfu DNA polymerase. The template DNA from each PCR reaction was restriction digested by 20 units of DpnI prior to transformation of E. coli DH5α (Stratagene). Plasmid DNA from the selected transformant was isolated using a Qiaprep mini-prep spin kit (Qiagen, Valencia, CA) and then digested with the appropriate restriction enzyme. A fragment of about 180 bases confirmed the insertion of the restriction site. The backbone vector was gel-purified using the Qiaquick gel extraction kit (Qiagen) and then religated in a 10-μl reaction using 3 units of T4 DNA ligase. The DNA sequences of the mutated LeuRS gene were confirmed by DNA sequencing (SeqWright, Houston, TX).

Plasmids expressing the wild-type and mutant E. coli LeuRS were used to transform E. coli BL21(DE3) cells and incubated at 37 °C overnight. A single cell transformant was transferred to a test tube containing 3 ml of Luria-Bertani (LB) broth with 100 μg/ml ampicillin and again incubated at 37 °C overnight. A 500-ml LB broth with 100 μg/ml ampicillin was inoculated using the entire 3-ml overnight culture and incubated by shaking at 300 rpm at 37 °C. When the A600 increased to between 0.6 and 0.8, the cells were induced with 1 mM isopropyl-β-d-thiogalactopyranoside for 2 h at 37 °C to express LeuRS. The recombinant yeast mitochondrial proteins were essentially expressed using the same procedures, with the exception that the transformed E. coli BL21(DE3) Codon Plus cells were grown and induced at 30 °C. The cells were then centrifuged at 6000 × g for 15 min in a Beckman J2-HS centrifuge with a JA-17 rotor. The cell pellets were collected and stored at −80 °C.

The frozen cell pellet was resuspended in 10 ml of buffer HA1 (10 mM Tris-HCl, 20 mM sodium P, pH 8.0, 100 mM NaCl, and 5% glycerol) and then sonicated at 60 A for 1 min using a Sonics Vibra Cell (Sonics, Newtown, CT). The sonication was repeated, and the cells were centrifuged in the Beckman JS-HS at 4 °C at 6750 × g for 15 min. Approximately 10 ml of lysate was added to 2 ml of His-Select™ resin that had been pre-equilibrated with buffer HA1. The resin was mixed at 4 °C for 1 h followed by centrifugation. The supernatant was removed and stored. The resin was washed once with 10 ml of HA2 buffer (10 mM Tris-HCl, 20 mM sodium P, pH 7.0, 500 mM NaCl, and 5% glycerol) followed by centrifugation and supernatant removal. The supernatant was relocated onto the resin and mixed at 4 °C for 30 min followed by centrifugation to remove the supernatant. The resin was washed a total of five times with 10 ml of HA2 buffer and centrifuged. The LeuRS with the six-histidine tag was eluted by mixing the resin with 1 ml of elution buffer (10 mM Tris-HCl, 20 mM sodium P, pH 8.0, 100 mM NaCl, 5% glycerol, and 200 mM imidazole). The purified protein was concentrated using a Centricon-50 (Amicon, Bedford, MA). The final concentration of the protein was determined using a Bio-Rad protein assay as described in the commercial protocol.

T7 RNA Polymerase Transcription of tRNA<sup>Leu</sup>—The plasmid pDTDNAleu14 containing the gene of E. coli tRNA<sup>Leu</sup> and plasmid p pymtDNAleu1 containing the gene of yeast mitochondrial tRNA<sup>Leu</sup> were digested with BstNI. The reaction was incubated overnight at 60 °C and the digested plasmid analyzed by agarose gel electrophoresis. The product of the BstNI digest was used as a template for in vitro transcription reactions containing 40 mM Tris-HCl, pH 8.1, 30 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.01% Triton X-100, 40 units of Prime RNase inhibitor (Eppendorf, Westbury, NY), 8 μg/ml pyrophosphatase (Sigma), 5 mM spermidine, 50 μg/ml bovine serum albumin, ATP, CTP, GTP, and UTP at 7.5 mM each, 80 μg/ml polyethylene glycol 8000, 60 μg/ml template, and 0.21 mg/ml T7 RNA polymerase (25, 26). The reaction was incubated at 42 °C for 6 h. The RNA product was precipitated by ethanol, washed twice with cold 70% ethanol by centrifugation, and vacuum-dried. The RNA was resuspended and purified on a denaturing 10% polyacrylamide gel that contained 8 M urea. The RNA bands were detected by UV shadowing, excised, crushed, and then incubated overnight with shaking at 37 °C in 0.5 M NH<sub>4</sub>OAC and 1 mM EDTA, pH 8.0. The procedure was repeated twice. The supernatant was collected, combined, and filtered through a 0.2-μm syringe filter. Butanol extractions were used to concentrate the RNA solution to 500 μl followed by the addition of 10 μl of 25 mg/ml glycogen and 1 ml of ethanol for an overnight precipitation at −80 °C. The tRNA pellet was collected by centrifugation, washed twice with 70% ethanol, and vacuum-dried. The concentration of tRNA was determined by absorbance at 260 nm using the extinction coefficient of 840,700 liters/mole/cm for E. coli tRNA<sup>Leu</sup> and 878,500 liters/mole/cm for yeast mitochondrial tRNA<sup>Leu</sup> (27). Purified tRNA<sup>Leu</sup> was denatured at 80 °C for 1 min followed by the addition of 1 mM MgCl<sub>2</sub> and quick-cooling on ice.

Aminoacylation Assays—Each aminoacylation reaction contained 60 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 4 μM folded tRNA<sup>Leu</sup>, 21 μM L-[<sup>3</sup>H]leucine (150 μCi/ml), and 50 mM enzyme. Kinetic rate constants were measured using six different tRNA<sup>Leu</sup> concentrations ranging from 0.3 to 25
The reactions were initiated by the addition of 4 mM ATP and then quenched at specific time points by transferring to filter pads (Whatman) that had been presoaked with 5% trichloroacetic acid. The pads were washed three times with cold 5% trichloroacetic acid and once with 70% ethanol, each washing for 10 min. The pads were dried by soaking in ether and then under an ultra-red lamp. Each pad was transferred into a 20-ml scintillation vial and quantitated using a Beckman LS6000IC scintillation counter.

Alternatively, acid gel analysis for each aminoacylation reaction was carried out using the conditions described above, except that 1.2 mM 1-[^14C]leucine (19 μCi/ml) was substituted for 1-[^3H]leucine. A 10-μl aliquot of the aminoacylation reaction was quenched with an equal volume of 30 mM NaOAC, pH 5.0, and 8 M urea. An 8-μl aliquot of each quenched reaction was separated by electrophoresis on a 10% acidic acrylamide gel that had been pre-equilibrated in 25 mM NaOAC, pH 5.0. Each sample was electrophoresed at 5 mA overnight or for ~20 h. The gel was dried and phosphorimaged using a Fuji BAS 1000 phosphorimaging screen (Fujifilm Medical Systems, Stamford, CT) and quantified by a Bio-Rad Molecular Imagier FX.

Inorganic Pyrophosphate (PPi) Exchange Assay — A 21-μl reaction contained 50 mM HEPES, pH 8.0, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM ATP, 1 mM [32P]PPi (2.5 μCi/ml) and 1 mM l-leucine was initiated with 100 nM enzyme. Aliquots were quenched on polyethyleneimine TLC plates (Scientific Adsortbents, Atlanta, GA) that had been pre-run in water. The TLC plates were developed in 750 mM KH₂PO₄, pH 3.5, and 4 M urea at 25 °C. Separated radiolabeled bands were detected by phosphorimaging as described above.

Hydrolytic Editing Assays — Hydrolytic editing assays were carried out at room temperature in 60 mM Tris (pH 7.5), 10 mM MgCl₂, and 0.8 μM [3H]lle-tRNA⁵⁵⁶. The reactions were initiated with 100 nM enzyme. At specific time points, 5-μl aliquots were spotted onto filter pads and washed as described previously (28–30).

Circular Dichroism — CD measurement was carried out using the Jasco J-720 spectropolarimeter. A sample containing 0.8 μM protein in 5 mM potassium Pi, pH 7.5, was measured in the far-ultraviolet region using an 0.1-cm path cell. Background signals from the cell and the buffer were subtracted from each spectrum.

RESULTS

A C-terminal Deletion Affects Splicing Complex Assembly and RNA Splicing Activity — Previously, we determined that yeast mitochondrial LeuRS and bl4 maturase simultaneously bind to the bl4 intron and stimulate splicing complex activity using an RNA-dependent two-hybrid assay (5). In addition, complementation assays have shown that a five-amino acid deletion of yeast mitochondrial LeuRS (18) and Mycobacterium tuberculosis LeuRS (6) abolish splicing activity but maintain protein synthesis. We used the RNA-dependent two-hybrid assay to test a five-amino acid truncation at the C terminus of yeast mitochondrial LeuRS (ΔC5) that was fused to the B42 activation domain and found that it forms a stable ternary complex with the bl4 intron and bl4 maturase. Fig. 1A shows that colonies develop a blue color because of an intracellular β-galactosidase reporter activity when the wild-type or five-amino acid C-terminal deletion is co-expressed with maturase and the bl4 intron. RNA isolated from the cells that exhibited ternary complex formation and splicing activities of yeast mitochondrial LeuRS wild-type and deletion mutants. A, RNA-dependent yeast two-hybrid system. The dark colonies have intracellular β-galactosidase activity that is representative of a two-hybrid response and indicates complex assembly of the yeast mitochondrial LeuRS, bl4 maturase, and bl4 intron. The colonies contained the bl4 intron as well as the yeast mitochondrial LeuRS and bl4 maturase that are expressed as B42 and LexA fusions, respectively. The top row shows a positive control using human isoleucyl-tRNA synthetase (IRS) and glutamic acid-proline tRNA synthetase (EPRS). The negative control is carried out in the absence of the bl4 intron. The plus and minus signs indicate the presence and absence of reporter activity. B, reverse transcription-PCR analysis of the splicing activity by yeast mitochondrial LeuRS and bl4 maturase using nuclear RNA extracts from the yeast two-hybrid cells. A band at 1.5 kb represents unspliced RNA including the flanking exons, and a band at 250 bp indicates ligated B4-B5 exons. The plus and minus signs indicate the presence and absence of bl4 intron, respectively.

Deletion of the C-terminal Domain Differentially Affects LeuRS from Different Origins — The co-crystal structure of T. thermophilus LeuRS with its tRNA bound was recently solved (14) (Fig. 2A). In the presence of tRNA, a folded C-terminal domain of 60 amino acids, which is linked to the canonical aminoacylation body of the LeuRS via a tether, binds to the D-loop of tRNA⁵⁵⁶. This domain was disordered in previous
LeuRS structures that lacked tRNA (15, 32). The co-crystal structure of T. thermophilus LeuRS also revealed RNA-protein interactions between the LeuRS C-terminal domain and the conserved G19:C56 tertiary corner of the tRNA (Fig. 2B). The primary sequence alignment of the LeuRS C-terminal domain exhibits significant homology (Fig. 2C). This suggests that a similarly folded domain is common to the LeuRSs and may be important to their function.

Based on this new crystal structure information, we designed deletion mutants to eliminate the entire C-terminal domain of E. coli and yeast mitochondrial LeuRSs and test for effects on their protein synthesis activities. The C-terminal domains of the E. coli and yeast mitochondrial enzymes were truncated after Glu797 and Ser828, respectively (Fig. 2C), leaving two amino acids from the end of a helix in the main body marked by Leu812 in the T. thermophilus LeuRS. Wild-type E. coli and yeast mitochondrial LeuRS as well as their deletion mutants were expressed with a fused N-terminal six-histidine tag and purified by affinity chromatography.

Deletion of the E. coli LeuRS C-terminal domain nearly abolished leucylation of E. coli tRNA^{Leu} (Fig. 3A). In contrast, when the C-terminal domain from yeast mitochondrial LeuRS was deleted, its aminoacylation activity was significantly enhanced compared with the wild-type yeast enzyme. Likewise, in aminoacylation reactions with yeast mitochondrial tRNA^{Leu}, both the E. coli and yeast mitochondrial LeuRS deletion mutants showed similar results (Fig. 3B). Deletion of the unique C-terminal domain increased the leucylation activity of yeast mitochondrial LeuRS but eliminated activity for E. coli LeuRS. Acid gel analysis of the charged E. coli and yeast mitochondrial tRNA products confirmed the opposing species-specific effects on aminoacylation activity (Fig. 3, C and D). These results support the proposition that the effects were not correlated to the cognate tRNA origins per se but broadly affected RNA interactions.

PPi exchange activity was measured directly by separating 32P-containing ATP and PPi on TLC plates. Each of the C-terminal deletion mutants exhibited similar PPi exchange activities compared with the respective wild-type enzymes (Fig. 4). Although the PPi exchange activity for the yeast mitochondrial LeuRS is weak compared with the E. coli enzyme, it is appreciable and overlaps for both the wild-type and C-terminal deletion mutant of yeast mitochondrial LeuRS. Thus, the active site catalytic core of the E. coli and yeast mitochondrial LeuRS that is responsible for amino acid activation appears to be largely unaffected by the deletion of the C-terminal domain.
We examined the yeast mitochondrial LeuRS wild-type and C-terminal domain deletion mutant by CD. Interestingly, Fig. 5 shows that the absence of the C-terminal domain yielded a protein that exhibits more α-helical character than the full-length wild-type LeuRS. Because the canonical class I core has similar activities for pyrophosphate exchange, we hypothesized that the structure of other more peripheral protein domains that are essential for transfer of the amino acid to tRNA may be impacted by the presence of the C-terminal domain in yeast mitochondrial LeuRS. It is also possible that these tRNA-binding domains cross-react in RNA splicing to interact with the group I intron. If this were the case, then evolutionary pressures could have forced the C-terminal domain or another region of the LeuRS that cooperates with the C-terminal domain to diverge in the yeast mitochondrial enzyme to accommodate its dual splicing and aminoacylation roles.

We also tested each of the deletion mutants for E. coli and yeast mitochondrial LeuRS for post-transfer editing activity. As found for aminoacylation, the E. coli LeuRS C-terminal domain deletion mutant showed significantly decreased hydrolysis activity of the mischarged E. coli or yeast mitochondrial Ile-tRNA\textsuperscript{Leu} compared with the wild-type enzyme (Fig. 6). In contrast, the yeast mitochondrial LeuRS C-terminal deletion showed a striking increase in hydrolysis activity of mischarged tRNA compared with its wild-type enzyme.

The post-transfer editing active site is located within the CP1 domain and is separated from the amino acid activation site by nearly 40 Å (Fig. 2A) (32). Movement of the tRNA between the aminoacylation and editing active sites is clearly accompanied by the leucine-specific C-terminal domain that interacts with the core of the tRNA. Deletion of the C-terminal domain profoundly affects both aminoacylation and amino acid editing activity and in a similar manner for each LeuRS. However, the effects oppose each other for LeuRSs from different origins. The presence of the leucine-specific C-terminal domain aids E. coli LeuRS while appearing to impede yeast mitochondrial LeuRS protein synthesis activities. It is possible that evolutionary pressures from the dual role of yeast mitochondrial LeuRS in RNA splicing resulted in species-specific differences.

Site-specific Mutational Analysis of the Leucine-specific C-terminal Domain—We mutationally scanned several conserved residues within E. coli LeuRS to identify specific sites that we hypothesized might be important to RNA-protein interactions between the C-terminal domain and tRNA\textsuperscript{Leu} (Fig. 2C and Table 1). These included conserved positively charged amino acids that could directly interact with the phosphate backbone of the tRNA. We also targeted specific sites based on the x-ray co-crystal structure of T. thermophilus LeuRS that appeared to be in close proximity to the bound tRNA.

As shown in Table 1, mutations of the targeted sites to either alanine or glutamic acid in E. coli LeuRS resulted in minimal, if any, effects on aminoacylation activities. Leu-tRNA\textsuperscript{Leu} was formed with similar catalytic efficiency (\(k_{\text{cat}}/K_m\)) for each of the mutants compared with wild-type LeuRS. The greatest change was a 5-fold reduction in \(k_{\text{cat}}/K_m\). The mutants outlined in Table 1 also failed to impact the post-transfer editing activity of mischarged tRNA molecules (data not shown). To more dramatically affect enzyme activity, we constructed double and triple mutants within the E. coli LeuRS, but these also showed only modest changes in \(k_{\text{cat}}/K_m\). Interestingly, a double mutant that reversed the charge of two conserved lysines (Lys\textsuperscript{846} and Lys\textsuperscript{853}) showed a significant increase in the \(k_{\text{cat}}\) value that was compensated for by an increase in the \(K_m\) for aminoacylation (Table 1).

We also introduced two mutations into yeast mitochondrial LeuRS that we postulated would disrupt interactions with the
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FIGURE 5. CD spectra of the LeuRS wild-type and C-terminal domain deletion. The yeast mitochondrial LeuRS wild-type and C-terminal deletion mutants are indicated by closed and open circles, respectively. Protein samples were prepared in 5 mM potassium Pi, pH 7.5.

Measurements

**FIGURE 6.** Post-transfer editing of Ile-tRNA_{Leu} by LeuRS wild type and ΔC deletion mutants. Decylation of mischarged E. coli (A) and yeast mitochondrial (B) Ile-tRNA_{Leu} is shown. Each reaction was carried out using 100 nM LeuRS and 0.8 μM [3H]Ile-tRNA_{Leu}. Wild-type E. coli LeuRS (Ec Wt); Y. **A**. LeuRS C-terminal deletion (Ec ΔC); Δ, yeast mitochondrial LeuRS C-terminal deletion (Ym ΔC); E, no enzyme.

tRNA based on the co-crystal structure of *T. thermophilus* LeuRS with its tRNA. However, mutations to alanine at Gln^{834} and Asn^{838} did not affect the protein synthesis activities (data not shown) as we have described above for the *E. coli* mutant enzymes. Thus, we propose that these conserved residues contribute primarily to folding and stabilization of the unique C-terminal domain. It is possible that interactions between the tRNA and LeuRS are not driven so much by specific atomic interactions as by more global features that conform to the overall bend at the corner of the L-shaped tRNA. In the case of yeast mitochondrial LeuRS, these RNA-protein interactions may have been optimized to accommodate RNA splicing.

**Chimeric Swapping of the C-terminal Domain Confers Wild-type-like Protein Synthesis Activities**—The C-terminal domains of yeast mitochondrial and *E. coli* LeuRS were swapped. The chimeric enzyme that comprised the *E. coli* enzyme fused to the yeast mitochondrial LeuRS C-terminal domain (*E. coli*ymCTD) retained significant aminoaacylation activity in the presence of either yeast or *E. coli* tRNA_{Leu} (Fig. 7A). Likewise, the *E. coli* C-terminal domain contains sufficient information to function in the context of the *E. coli* LeuRS to support its enzyme activities. Introduction of the *E. coli* C-terminal domain into the yeast mitochondrial LeuRS failed to significantly alter aminoaacylation or editing activities. We hypothesize then that the C-terminal domain functions in concert with other domains or portions of the LeuRS enzyme, as postulated based on CD structural analysis above, to support both the yeast mitochondrial LeuRS RNA splicing and protein synthesis activities.

**DISCUSSION**

LeuRS is essential to group I intron splicing and protein synthesis within the yeast mitochondria. Previous experiments showed that short C terminus truncations abolished splicing activity, whereas protein synthesis activity is maintained (6, 18). We determined that a short truncation of just five amino acids, which fails to complement yeast null strains, can form a ternary complex with the bI4 intron and the bI4 maturase splicing partner. However, in the presence of the LeuRS truncation, the complex is not competent to induce splicing activity either in complementation assays or under yeast two-hybrid conditions.

The x-ray co-crystal structure of *T. thermophilus* LeuRS bound to tRNA shows that a unique C-terminal domain interacts directly with the tRNA. We hypothesize that this conserved RNA-binding domain in yeast mitochondrial LeuRS also interacts with the bI4 intron and has co-evolved to accommo-
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Enzymatic activities of chimeric LeuRS with swapped C-terminal domains. Aminoacylation assays were carried out using 4 μM E. coli (A) and yeast mitochondrial (B) tRNA<sup>Leu</sup> and 50 nM enzyme. C, post-transfer amino acid editing activity was measured using 100 nM LeuRS and 0.8 μM [3H]Ile-tRNA<sup>Leu</sup>. , wild-type E. coli LeuRS (Ec Wt); , wild-type yeast mitochondrial LeuRS (Ym Wt); , E. coli LeuRS with yeast mitochondrial C-terminal domain chimera (Ec ymCTD); , yeast mitochondrial LeuRS with E. coli C-terminal domain chimera (Ym EcCTD); , no enzyme.

FIGURE 7. Enzymatic activities of chimeric LeuRS with swapped C-terminal domains. Aminoacylation assays were carried out using 4 μM E. coli (A) and yeast mitochondrial (B) tRNA<sup>Leu</sup> and 50 nM enzyme. C, post-transfer amino acid editing activity was measured using 100 nM LeuRS and 0.8 μM [3H]Ile-tRNA<sup>Leu</sup>. , wild-type E. coli LeuRS (Ec Wt); , wild-type yeast mitochondrial LeuRS (Ym Wt); , E. coli LeuRS with yeast mitochondrial C-terminal domain chimera (Ec ymCTD); , yeast mitochondrial LeuRS with E. coli C-terminal domain chimera (Ym EcCTD); , no enzyme.

date both of the critical functions of the enzyme in splicing group I introns and aminoacylation of tRNA. We compared the protein synthesis activities using C-terminal deletion mutants of both E. coli and yeast mitochondrial LeuRS. In the absence of the C-terminal domain, both aminoacylation and editing activities of the E. coli LeuRS were abolished. Deletion of the C-terminal domain from various LeuRSs also impacts their aminoacylation activities (14, 16, 17). In striking contrast, deletion of the yeast mitochondrial C-terminal domain clearly enhanced aminoacylation and editing activities. It is likely that as evolutionary pressures recruited and optimized LeuRS to carry out a secondary role in RNA processing, a region of the LeuRS that impacts the C-terminal domain, either directly or indirectly via an RNA mediator, adapted to maintain critical levels of aminoacylation for protein synthesis while optimizing splicing activity to express essential mitochondrial genes. This adaptation has resulted in functional divergence of the LeuRS C-terminal domain.

Interestingly, the presence of the yeast mitochondrial C-terminal domain appears to impede significantly the editing activity in vitro, which could threaten the fidelity of protein synthesis. It is possible that the benefit of splicing the cob and cox1α genes in yeast mitochondria may outweigh the cost of an elevated level of misincorporation of amino acids during translation. Examples of editing aminoacyl-tRNA synthetases from origins that lack editing activities include the human cytoplasmic ProRS, human mitochondrial LeuRS, and yeast mitochondrial PheRS. Each fails to hydrolyze mischarged tRNAs or mis-activated adenylates (33–35). For the human enzymes, it has been proposed that enhanced discrimination within the aminoacylation active site combined with low intracellular concentrations of noncognate amino acids that are below the K<sub>m</sub> of the enzyme apparently suffice to achieve fidelity levels that maintain mitochondrial and/or cellular functions.

Previously, LeuRSs that originated from varied origins, including bacterial and human mitochondria, were shown to effectively complement yeast null strains (6). The C-terminal domains for other LeuRSs are clearly important for aminoacylation (14, 16, 17). Although the yeast mitochondrial LeuRS is dependent on the C terminus for splicing activity, the presence of the C-terminal domain impedes its protein synthesis activities. Thus, it is clear that variations within LeuRS that impact the C-terminal domain can shift the aminoacylation, editing, and splicing function of LeuRS in a dramatic way that ranges from significant increases to limiting activity. These evolutionary shifts may have played an important role in conferring an essential secondary activity in splicing to yeast mitochondrial LeuRS, in addition to its original role in protein synthesis.

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