Combinatorial Analysis of Loop Nucleotides in Human Mitochondrial tRNA$^{Leu(UUR)}$ †

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ABSTRACT: A series of disease-related mutations are known to affect the hs mt tRNA$^{Leu(UUR)}$ gene, and the molecular-level properties of this tRNA may underlie the effects of pathogenic sequence changes. A combinatorial approach has been used to explore the importance of the D, TΨC, and anticodon loops of hs mt tRNA$^{Leu(UUR)}$ in the structure and function of this molecule. A tRNA library was constructed with 20 randomized nucleotides in the loop regions of hs mt tRNA$^{Leu(UUR)}$, and tRNA variants that were aminoacylated by hs mt LeuRS were isolated using an in vitro selection approach. Analysis of 26 selected sequences revealed that a stabilized anticodon stem significantly enhances aminoacylation activity. However, anticodon loop nucleotides were not conserved in the active sequences, indicating that this region of hs mt tRNA$^{Leu(UUR)}$ is not involved in recognition by LeuRS. Within the D and TΨC loops, only two nucleotides conserved their identities, while new sequences were selected that likely mediate interloop interactions. The results indicate that hs mt tRNA$^{Leu(UUR)}$, which is known to have structurally weak D and anticodon stems, benefits functionally from the introduction of stabilizing interactions. However, the locations of individual nucleotides that govern discrimination of this tRNA by hs mt LeuRS still remain obscure.

Transfer RNAs (tRNAs)[1] are essential components of the translational machinery and are responsible for delivering amino acids to the ribosome and mediating their insertion into proteins as dictated by mRNA codons (*1*). High-fidelity protein synthesis depends on the accurate recognition and aminoacylation of tRNAs by cognate aminoacyl-tRNA synthetases (aaRSs) (*2*). To achieve selective binding of tRNAs, these enzymes rely on the presence of identity elements—specific sets of nucleotides that provide contacts for molecular recognition. Identity elements are often located in the acceptor stem and the anticodon loop of tRNAs, but can be found in other regions (*3*). In addition to sequence-specific recognition facilitated by contacts to base functionalities, shape-selective recognition can be achieved for unique tRNA backbone conformations (*3*).

While in some organisms, like *E. coli*, complete sets of identity elements are known for all tRNA-synthetase pairs (*3*), mitochondrial (mt) systems are poorly understood. In particular, the tRNAs of mammalian mitochondria often feature significant deviations from canonical (bacterial and cytosolic) tRNA sequences (*4*). Mt tRNAs typically have fewer GC base pairs, more AU and GU base pairs, and a higher frequency of unstable base mismatches. In addition, they commonly have shorter D and TΨC loops and lack many of the nucleotides typically involved in tertiary interactions (*4*). Mammalian mt tRNAs therefore represent novel systems that follow different rules to achieve stable, folded conformations. Moreover, the recognition of these tRNAs by cognate aaRSs may involve different sets of contacts than those identified in canonical systems.

The tRNAs of human mitochondria are of particular interest because disease-related mutations appear within the genes encoding these molecules (*5*−*7*). During the last two decades, over 90 pathogenic tRNA mutations have been identified within the human mt genome (*8*). The hs mt tRNA$^{Leu(UUR)}$ gene is a hot spot for pathogenic mutations, with 20 presently documented (*8*). Effects of several prevalent mutations have been studied at the cellular and molecular levels and appear to affect many mt processes, including respiration, protein synthesis, tRNA processing, post-transcriptional modifications, and aminoacylation (*9*, *10*).

The hs mt tRNA$^{Leu(UUR)}$ has a variety of unique structural features that may impact its function and influence the effects of pathogenic mutations. This tRNA contains most of the nucleotides that permit formation of canonical tertiary structural elements (Figure 1A). However, it has an unstable anticodon stem, which consists of four A−U base pairs, and a loosely structured D stem that contains two Watson−Crick base pairs, an A−C mismatch and a G−U base pair. As a result, the D/anticodon branch of the hs mt tRNA$^{Leu(UUR)}$ is thermodynamically unstable, and it has been shown by enzymatic probing that the structure of this domain is highly dynamic (*11*−*13*).

While the metastable structure of hs mt tRNA$^{Leu(UUR)}$ appears to follow the canonical folding pattern, the identity elements within this sequence that impart efficient and

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[1] Abbreviations: hs mt, human mitochondrial; tRNA, transfer ribonucleic acid; D, dihydrouridine; aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; WT, wild type.

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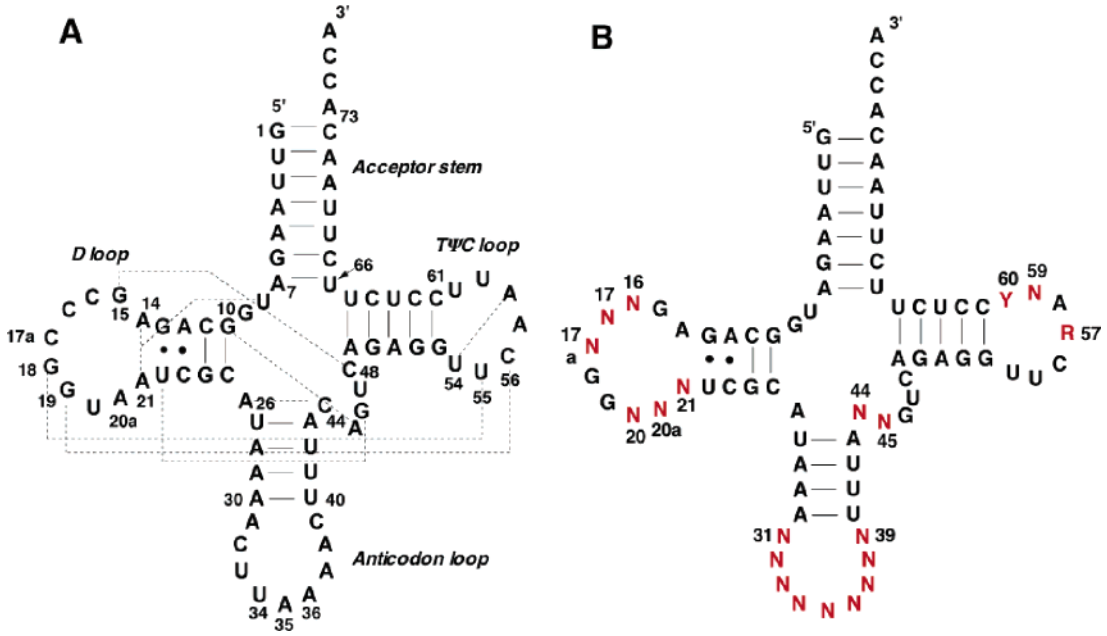

FIGURE 1: (A) Predicted secondary structure of hs mt tRNA$^{Leu(UUR)}$. Expected tertiary interactions are indicated with dotted lines. Positions are numbered according to ref *37*. Base modifications are not shown. (B) Design of combinatorial library based on the hs mt tRNA$^{Leu(UUR)}$ sequence. Randomized positions are shown in red. N = any nucleotide, R = purine, Y = pyrimidine.

specific aminoacylation by hs mt LeuRS remain somewhat obscure. The nucleotides used for recognition of tRNA^{Leu} by LeuRS have been identified in several other systems (*14*−*21*). Interestingly, studies of tRNA^{Leu} from eubacteria, archea, yeast cytoplasm, plant cytoplasm, and human cytoplasm have revealed idiosyncratic aminoacylation determinants that are not conserved through evolution. The only common identity element present in all systems appears to be the universally conserved discriminator base A73 (*22*). Other recognition elements are located in the acceptor stem [human cytoplasm (*14*)], long variable arm [*Haloferax volcanii*, human cytoplasm (*14*, *15*)], anticodon loop [*Saccharomyces cerevisiae* (*16*)], and D loop [human cytoplasm (*14*)]. In *Escherichia coli*, leucylation is largely dependent on several nucleotides involved in tertiary interactions within the elbow region of tRNA (*17*−*21*). For hs mt tRNA^{Leu}, previous studies suggest that A73 and A14 are identity elements (*22*), although it is difficult to deconvolute the role of A14 in tRNA tertiary structure from its function in recognition by LeuRS (*23*). Footprinting of hs mt tRNA$^{Leu(UUR)}$ with LeuRS suggests contacts with the acceptor stem, anticodon domain, and the D loop (*11*); thus a dispersed set of nucleotides may serve as determinants in this tRNA.

In the present study, we explore the roles of the D, TΨC, and anticodon loops in the structure of hs mt tRNA$^{Leu(UUR)}$ and interactions between these domains and the cognate aaRS. To investigate the role of many nucleotides simultaneously, we constructed a combinatorial tRNA library featuring randomized positions within the D, TΨC, and anticodon loops of hs mt tRNA$^{Leu(UUR)}$ and selected RNA molecules that were aminoacylated by hs mt LeuRS. Analysis of selected sequences revealed that while the identity of anticodon loop nucleotides did not affect aminoacylation, a stabilized anticodon stem significantly enhanced activity, indicating that the structure of this region of hs mt tRNA$^{Leu(UUR)}$ was more important for recognition by LeuRS than its precise sequence. Within the D and TΨC loops, a subset of nucleotides was retained; however, new sequences were selected that likely mediate interloop interactions. The results obtained indicate that efficiency of aminoacylation for this structurally unstable tRNA is enhanced by stabilizing interactions; the identity of nucleotides critical to the recognition and discrimination of hs mt tRNA$^{Leu(UUR)}$ by LeuRS still remains obscure.

MATERIALS AND METHODS

Preparation of tRNA Library. Library DNA (5′-TGGTG-TTAAGAAGAGGRNTYGAACCTCTGACNNTAAANNN-NNNNNNTTTATGCGANNNCCNNNCTCTGCCATCTT-AACTATAGTGAGTCGTATTAGGATCC-3′; R = purine, Y = pyrimidine) was synthesized chemically (BioCorp Inc, Montreal, Canada), purified using 12% denaturing PAGE, and amplified by PCR (100 pmols each; PCR primer, 5′-C C A T A G G A T C C T A A T A C G A C T C A C T A T-AGTTAAGATGGCAGAG-3′; RT primer, 5′-TGGTGT-TAAGAAGAGG-3′) with 200 μM dNTPs and 10 units of *Taq* DNA polymerase (New England Biolabs) in 100 μL total reaction volume. For each PCR reaction, 100 ng (six copies of the library) of gel-purified template were used. Cycling was performed in a RoboCycler Gradient 96 (Stratagene). Multiple PCR reactions were pooled, phenol/chloroform extracted, and ethanol-precipitated. The initial tRNA pool was prepared by in vitro transcription with T7 RNA polymerase as described previously (*13*) using the PCR product as a template. After transcription, the tRNA pool was purified by 12% PAGE, electroeluted, ethanol-precipitated, and resuspended in DEPC-treated 0.5× TE buffer (5 mM Tris-HCl (pH 8)/0.5 mM EDTA). tRNA concentrations were determined by measuring absorbance at 260 nm.

Aminoacylation. Prior to aminoacylation, tRNA was annealed by incubation for 5 min at 70 °C, addition of 10 mM $MgCl_2$, and cooling on ice. Purification of hs mt LeuRS was performed as described (*23*, *24*). Enzyme concentration was determined using the Bradford reagent (Bio-Rad) and a BSA standard. Aminoacylation was performed at 37 °C in 50 mM

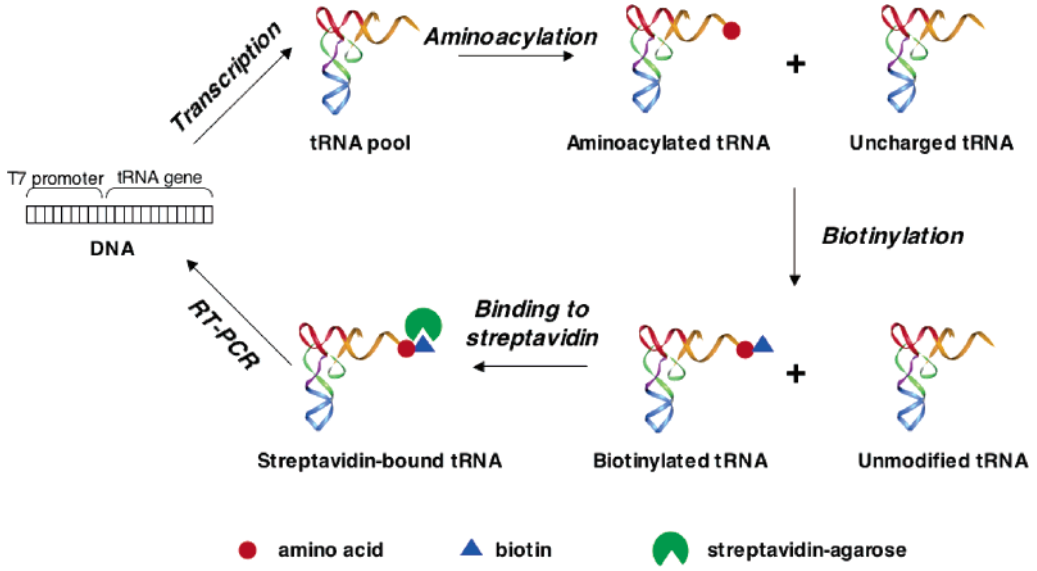

FIGURE 2: Summary of the procedure for selection of aminoacylated tRNAs (adapted from ref *26*). Conditions for each step are described in the Materials and Methods section.

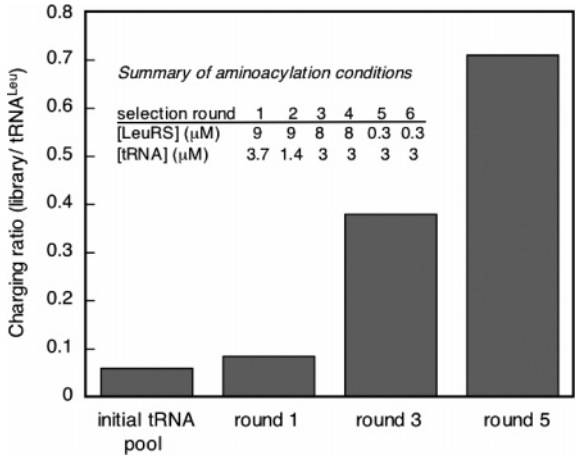

FIGURE 3: Plateau charging levels of tRNA pools throughout selection. Values are relative to the plateau charging of WT hs mt tRNA$^{Leu(UUR)}$. Determination of plateaus for each tRNA pool was performed under aminoacylation conditions for the next round of selection (see the inset), with exception of round 5, when 3 μM tRNA pool and 3 μM LeuRS were used. (Inset) Summary of aminoacylation conditions for each selection round.

HEPES−KOH, pH 7.6, 25 mM KCl, 100 μM spermine, 0.2 mg/mL BSA, 2.5 mM ATP, 7 mM $MgCl_2$, 100 μM leucine, 4.6 μM [3,4,5-^{3}H]leucine (173 Ci/mmol, Perkin-Elmer), 30 nM to 9 μM LeuRS, and 1−4 μM tRNA. Aliquots (2 μL) were removed from the reaction at regular time intervals and spotted on Whatman circles treated with 5% trichloroacetic acid (TCA), and the amount of aminoacylated tRNA was determined by counting the amount of specifically incorporated [^{3}H]leucine after nonincorporated leucine was washed away with 5% TCA.

Selection. Aminoacylation of tRNA pools during selection was performed under conditions shown in the inset in Figure 3. Samples were then extracted with acidic phenol/chloroform (pH 4.7), nonincorporated leucine was removed by two subsequent filtrations using Microcon-10 filters (Millipore), and the tRNA was ethanol-precipitated.

Biotinylation. For biotinylation, tRNA was resuspended in 10 μL of ice-cold 0.3 M HEPES (pH 5.3), and 10 μL of 0.3 M HEPES (pH 9.5) and 10 μL of 10 mg/mL fresh aqueous solution of Sulfo-NHS-SS-biotin (Pierce) were added. Biotinylation reactions were incubated for 1 h on ice and then diluted in 0.2 M NH_4OAc (pH 5.2)/1% MeOH. Excess biotin was removed by three subsequent filtrations with Microcon-10, after which the tRNA was ethanol-precipitated.

Binding to Streptavidin−Agarose. Streptavidin-coated agarose beads (100 μL) (Immunopure immobilized streptavidin, Pierce) were washed extensively with binding buffer (1 M NaCl/10 mM HEPES−KOH, pH 7.5). Biotinylated tRNA was resuspended in 100 μL of binding buffer, added to the streptavidin−agarose beads, and incubated for 30 min at room temperature with gentle shaking. Beads were rinsed several times with binding buffer and then binding buffer containing 100 mM DTT was added to cleave tRNA from the beads, and the reaction was incubated for 30 min at room temperature. tRNA was recovered by filtration through a Spin-x filter (Corning) and ethanol precipitation.

Reverse Transcription and PCR. To reverse transcribe selected tRNA sequences, tRNA was resuspended in the reverse transcriptase buffer, 100 pmol of RT primer was added, the reaction was heated for 1 min at 90 °C and cooled quickly to 37 °C, and dNTPs (200 μM) and 400 units of M-MuLV reverse transcriptase (New England Biolabs) were added. The reaction was allowed to proceed for 1 h at 37 °C, and then 5 μL of the reverse transcription reaction was used as a cDNA template for PCR amplification, which was performed as described above. The products of five PCR reactions were combined to prepare a new tRNA pool for the next round of selection.

Cloning and Characterization of Selected tRNAs. After the sixth round of selection, PCR was performed using a cloning primer (5′-GTTGGCTGCAGCCTGGTGTTAA-GAAGAGG-3′) instead of the RT primer, and the products were cloned into the pUC18 plasmid using *Bam*HI and *Pst*I restriction sites. During cloning, a T7 RNA polymerase promoter sequence was introduced upstream of the tRNA genes, and the *Mva*I restriction site was introduced at the 3′ end of tRNA genes to allow in vitro transcription of tRNAs

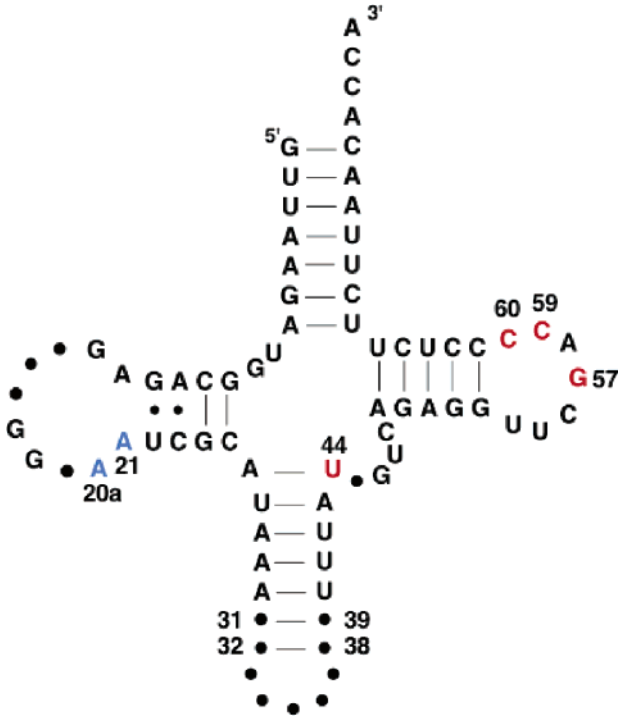

FIGURE 4: Consensus sequence of selected tRNAs. Conserved nucleotides that are the same as in WT are shown in blue, those different from the WT are shown in red, and those that are not conserved are shown as filled circles. Additional base pairs 31−39, 32−38, and 26−44 in the anticodon stem are indicated.

with the correct 5′ and 3′ termini. tRNA genes from 26 clones were sequenced by Genewiz, Inc. (North Brunswick, NJ).

To investigate aminoacylation activity of selected variants, tRNA samples were prepared by in vitro transcription with T7 RNA polymerase using plasmid DNA digested with *Mva*I (Ambion) as a template and purified as described above. For each tRNA sequence, at least two transcription reactions were performed. Aminoacylation was analyzed with 3 μM tRNA and 100 nM LeuRS in a total volume of 14 μL. Data from two to four trials were used to calculate the initial rate of aminoacylation (V_0) for each variant.

Construction of Hybrid tRNA Constructs. Hybrid tRNA constructs (Figure 5) were prepared by ligating overlapping oligonucleotides into *Bam*HI and *Pst*I sites of pUC18 plasmid, as described previously (*12*).

Native Gel Electrophoresis. Samples containing 300 ng of tRNA were annealed as described above, loaded on a 12% polyacrylamide gel in 0.5× TB buffer [45 mM Tris (pH 7.5), 45 mM boric acid], and stained with ethidium bromide. Gels were imaged with a UVP Imaging System.

RESULTS

Selection of Active hs mt tRNA$^{Leu(UUR)}$ Sequences. To probe how the D, TΨC, and anticodon loop nucleotides of hs mt tRNA$^{Leu(UUR)}$ influence its aminoacylation by the hs mt LeuRS, we constructed a library of tRNAs where several positions in each loop were randomized (Figure 1B). To preserve the tertiary fold of the tRNA, randomization was mostly restricted to nucleotides that are not involved in the formation of tertiary interactions. Exceptions to this constraint include position 21, which can participate in the formation of the 8−14−21 base triple; position 44, which usually forms a noncanonical base pair with nucleotide 26; and position 45, which can participate in the base triple 10−25−45 (*25*). Eighteen nucleotides were randomized completely, while positions 57 and 60 in the TΨC loop were only partially randomized and restricted to purines and pyrimidines, respectively. The resulting library had a complexity of 2.75 × 10^{11} variants.

To select tRNA variants that could be aminoacylated with hs mt LeuRS, it was necessary to use a procedure that would separate aminoacylated from nonaminoacylated tRNAs. Such a system has been described (*26*, *27*) and is based on selective biotinylation of aminoacylated tRNAs (Figure 2). Biotinylated tRNAs are captured on streptavidin resin, and corresponding DNA sequences are obtained by reverse transcription and PCR.

We applied six rounds of selection to the initial tRNA library. Since the starting tRNA pool was very inefficiently charged (Figure 3), a high concentration of enzyme was used during the aminoacylation step for the first rounds of selection (see inset in Figure 3). By round 3, the plateau charging level (maximum charging obtained with high enzyme concentration) of the tRNA pool had increased significantly. Selection was performed with a high LeuRS concentration for one more round, and the enzyme concentration was decreased to $^1/_{10}$ of the tRNA concentration for the last two rounds. This change in the selection conditions was made in order to favor tRNA variants with higher affinity for the enzyme. By the end of selection process, the plateau charging level of the tRNA pool had reached 70% of that observed with WT tRNA$^{Leu(UUR)}$. The selection was halted at this point to provide the opportunity to analyze new sequences that might display comparable activities to the WT tRNA$^{Leu(UUR)}$.

Analysis of Selected Sequences. After the sixth round of selection, the tRNA library was cloned and the variants were sequenced. Sequences of 26 individual tRNAs were obtained (Table 1). Each of the sequences identified was unique, and one (sequence 25) was identical to WT hs mt tRNA$^{Leu(UUR)}$. All other sequences presented significant deviations from the WT sequence in the randomized regions. A number of sequences also had nonprogrammed mutations, typically insertions of an additional nucleotide in the anticodon loop or insertion/deletion of nucleotides in the variable loop. PCR amplification with relatively low-fidelity *Taq* DNA polymerase was the likely source of these nonprogrammed mutations.

The longest stretch of randomized nucleotides was located in the anticodon loop of tRNA and included positions 31 and 39 flanking the loop. Within this region, none of the WT nucleotides were conserved among selected variants. However, the first and last randomized positions, 31 and 39, always covaried to form a Watson−Crick base pair (Figure 4). This extra base pair was formed in > 90% of sequences selected (exceptions are sequences 25 and 13). In 60% of the sequences, a GC or CG base pair was present, but AU or UA pairs also appeared in ∼25% of the sequences, and in one case a UG pair was observed (sequence 24).

Positions 31 and 39 form a Watson−Crick base pair in most tRNAs. However, in both hs mt tRNA^{Leu} isoacceptors there is a mismatch between these nucleotides in the anticodon stem, which is considered to be a weak point in the fragile structure of this tRNA (*11*−*13*). The presence of the 31−39 base pair in selected tRNA variants can be

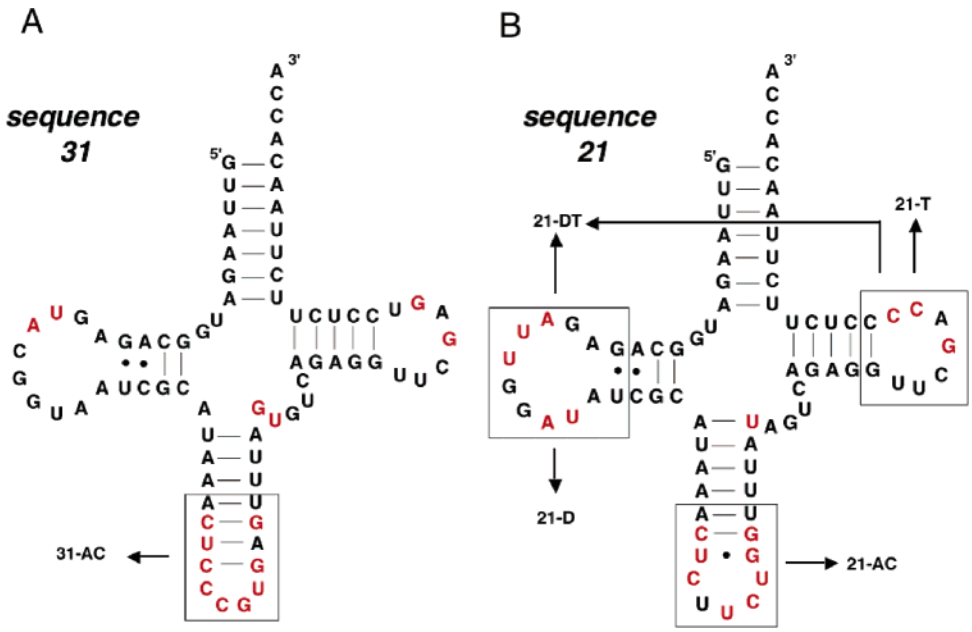

FIGURE 5: Design of hybrid tRNA constructs based on sequences 31 and 21. Nucleotides different from WT are shown in red. (A) Secondary structure of variant 31. Anticodon sequence (boxed) was introduced into hs mt tRNA$^{Leu(UUR)}$ to produce construct 31-AC. (B) Secondary structure of variant 21. Regions that are boxed were introduced into hs mt tRNA$^{Leu(UUR)}$ to produce constructs 21-AC, 21-D, 21-T, and 21-DT.

considered a stabilizing structural feature that makes the anticodon stem more comparable to canonical tRNAs. Indeed, introduction of a G31−C39 base pair into a WT tRNA$^{Leu(UUR)}$ transcript was previously shown to increase its aminoacylation rate 5.5-fold (*11*).

Moreover, in more than 75% of selected sequences, another additional base pair is formed between positions 32 and 38, further stabilizing the anticodon stem. CG or GC base pairs are found in 60% of sequences, and UA or UG pairs also appear in several sequences. Formation of the 32−38 base pair would result in a five-nucleotide anticodon loop, instead of the canonical size of seven nucleotides (*25*). In theory, shortening of the anticodon loop would interfere with the ability of tRNA to function in protein synthesis. However, the only criterion for selection of tRNA in our in vitro system was charging by hs mt LeuRS, which appears to tolerate a shorter anticodon loop. Nine of the isolated sequences can even potentially form a third base pair (33−37), which would leave only three nucleotides in the anticodon loop. In addition to extra base pairs, two of the most active sequences (31 and 26), as well as two others (22 and 11) have an insertion of a nucleotide in the anticodon loop: instead of nine bases, their randomized anticodon regions contain 10 nucleotides. It is clear that significant changes in the structure of the anticodon stem do not have a strongly negative effect on aminoacylation.

On the other end of the anticodon stem, position 44 was also randomized. Though this position is occupied by a C in hs mt tRNA$^{Leu(UUR)}$, in 75% of selected sequences it is a U, which can make an AU base pair with A26, thus additionally stabilizing the anticodon stem (Figure 4). In contrast, the adjacent randomized position 45 does not show any conservation among selected tRNAs.

Six positions (16, 17, 17a and 20, 20a, 21) were randomized in the D loop. Nucleotides from the WT sequence were only found at two positions with significant frequency: A21 and, to a lesser extent, A20a. At other randomized positions (17, 17a and 20) WT nucleotides were found only in about 30% of sequences, and the WT C16 was present only in one sequence. A21 is believed to be involved in an 8−14−21 base triple (*25*). The preservation of A at position 21 in many of the selected sequences may reflect the importance of this tertiary interaction.

The TΨC loop was randomized at positions 57, 59, and 60. All three positions were highly conserved among selected variants, but their identity was different from WT hs mt tRNA$^{Leu(UUR)}$ (Figure 4). In almost all selected sequences nucleotide 57 is changed from A to G, and nucleotides 59 and 60 are changed from U to C.

Analysis of the selected sequences produced the consensus pattern shown in Figure 4. Major differences from the WT hs mt tRNA$^{Leu(UUR)}$ are the presence of additional base pairs in the anticodon stem (31−39, 32−38, and 26−44) and nucleotides G57, C59, and C60. Only two sequences deviate substantially from this pattern: sequence 25, which is identical to WT sequence, and sequence 13, which cannot form any additional base pairs in the anticodon stem and also contains an A57.

The presence of additional base pairs in the anticodon stem should result in a conformation different from that of WT tRNA$^{Leu(UUR)}$. Indeed, when migration of selected variants was analyzed by native PAGE (see Supporting Information), it was found that all variants migrate much faster than WT tRNA$^{Leu(UUR)}$, indicating that the stabilizing sequence changes promoted the formation of a more folded, compact conformation (*28*).

Aminoacylation Activities of Selected Variants. Aminoacylation of selected variants was studied by measuring the initial rate of aminoacylation of tRNA transcripts using identical conditions for all sequences (3 μM tRNA and 100 nM LeuRS). All tRNAs show a decrease in aminoacylation rate compared to the WT transcript, which ranged from 3-fold to almost 50-fold (Table 1), and featured a high number of sequences at the low end of this range. The relatively low

Table 1: Sequences of TRNAs Selected from the Library and Initial Aminoacylation Rates[a]

	D loop	D stem	Anticodon stem	Anticodon loop	Anticodon stem	Variable loop	TΨC stem	TΨC loop	V_0 (fmol/min)
Position	14 15 16 17 17a18 19 20 20a 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 - 36 37 38 39 40 41 42 43 44 45 - 46 47 48 - 49 50 51 52 53 54 55 56 57 58 59 60								
tRNA^{Leu} (WT)	A G C C C G G U A A U C G C A U A A A A C U U A - A A A C U U U A C A - G U C - A G A G G U U C A A U U								470 ± 60
Selected sequences									
25	WT hs mt tRNA$^{Leu(UUR)}$								
31	U A C U A A			C U C C C G U G A G		G U		G G U	160 ± 30
26	G C U C A A			A U G A A A C A C U		U A		G C C	120 ± 20
30	G A U C A A			G C A C A U C G C		U C		G C C	100 ± 20
5	G C A U A A			G C G C U A A G C		G U		G C C	82 ± 9
21	A U U A U A			C U C U U C U G G		U A		G C C	72 ± 6
23	G C U A A A			U C U U U A U G A		A A A		G C C	50 ± 20
12	U C G U A C			G U U C U U A A C		U G U C		G G U	43 ± 7
29	G C A C A A			C G C U A A U C G		U U		G C C	41 ± 9
32	A U U A U A			C U U C U U C C G		U U		G C C	40 ±10
15	A U U A U A			C G U C G U A C G		U C		G C C	32 ± 8
33	A A U A U A			G C C C A A C A C		C U		G C C	30 ± 4
13	U A C U C A			C G C U G U G A C		C U -		A C C	30 ± 10
22	A C U A A U			C C A C G U U G G G		U U		G C C	28 ± 7
11	A U U A A A			A C C G U U A C G U		U C		G C C	25 ± 6
4	A U U U U U			C C U U C G A G G		U A		G C C	16 ± 2
2	A U U A A A			G U U A A A A A C		U C		G C C	15 ± 3
17	A G C A A A			U C C C C G A G A		U U		G C C	12 ± 2
16	U U A C A C	-		G C G A C C C G C		G U		G G U	10 ± 1
20	U U A A A A			A C A A U U U G U		U C		G C C	11 ± 2
24	A C C G U U			U U A G U A U G G		U G		G C C	10 ± 1
8	U A A U A C			C C U U C A G G G		U G		G C U	-[b]
9	G A U U A G A			G C A C U A A G C		U A -		G C C	-[b]
14	A C U A U A			U C C C A U U G A		U C A	A	G C C	-[b]
19	G U U C U A U			A C U C C U U G U		U A	A	G C C	-[b]
7	A U U A U A			G C U A A A G A C		U U		G C C	nd[c]

[a] Randomized regions are underlined in the hs mt tRNA$^{Leu(UUR)}$ sequence. For the library variants, only the sequences of the randomized regions and mutations outside these regions are shown. Variants are ranked according to their aminoacylation activity. Initial rate of aminoacylation (V_0) was measured using 3 μM tRNA and 100 nM enzyme for all variants. At least two tRNA transcripts were analyzed for each tRNA variant. For each transcript, two to four aminoacylation assays were performed. Numbers are represented as mean ± standard error from all experiments. [b] No aminoacylation was detected under test conditions. [c] Aminoacylation of variant 7 was not studied because of the deletion of the *Mva*I digestion site that prevented proper transcription.

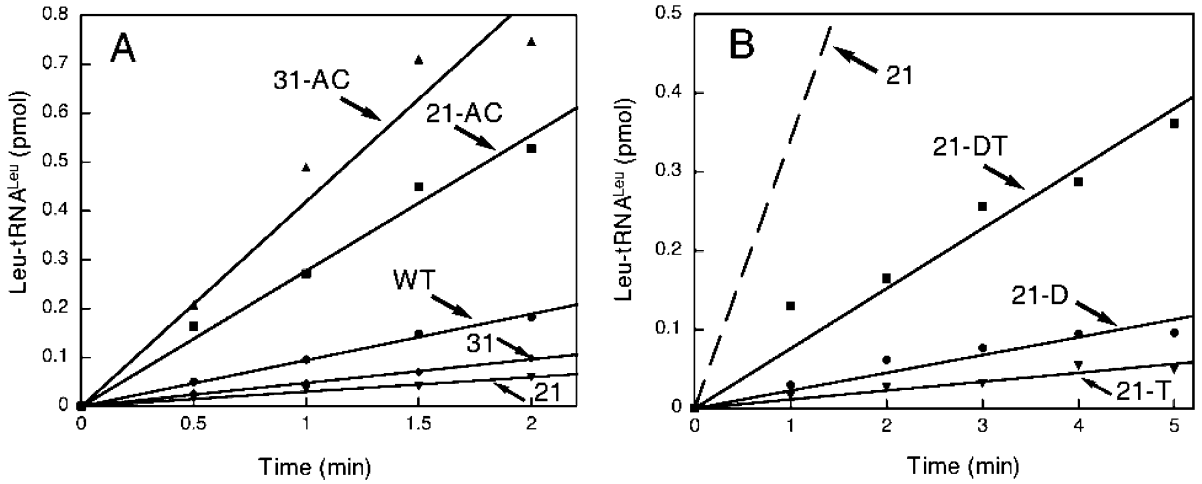

FIGURE 6: Aminoacylation of hybrid tRNA constructs. (A) Aminoacylation of WT hs mt tRNA$^{Leu(UUR)}$ (●), sequences 31 (◆) and 21 (▼), and constructs 31-AC (▲), and 21-AC (■) by hs mt LeuRS. Aminoacylation was performed with 2 μM tRNA and 30 nM LeuRS. (B) Aminoacylation of tRNA sequence 21 (dotted line) and of constructs 21-D (●), 21-T (▼), and 21-DT (■) by hs mt LeuRS. Aminoacylation was performed with 3 μM tRNA and 300 nM LeuRS.

charging levels observed do not contradict the data in Figure 3, which shows high plateau charging levels at the end of selection, since the postselection plateau charging levels were determined with much higher enzyme concentrations (3−9 μM) and longer incubation times.

Interestingly, sequences 8, 9, 14, and 19 did not show detectable aminoacylation under standard conditions. We noticed, however, that all these variants contain mutations destabilizing tRNA structure. Sequences 9 and 19 both contain an A14G mutation, a well-known pathogenic mutation that disrupts the 8−14 tertiary contact and has been shown to have a detrimental effect on aminoacylation of hs mt tRNA$^{Leu(UUR)}$ (*23*). Sequences 14 and 19 contain point mutations (G53A and G50A, respectively) introducing mismatches into the TΨC stem. We hypothesize that these mutations arose during the last PCR amplification after the last round of selection and that they inhibit aminoacylation by destabilizing tRNA structure. For example, clone 8 folds into a very stable alternative secondary structure with a long D stem (*29*), which may grossly interfere with LeuRS recognition. While it seems surprising that such a high frequency of mutations could occur, this is the most likely explanation, as the least active sequences are clearly related to the more active variants. The fact that mutations are also observed in selected sequences at sites that were not randomized indicates that the fidelity of *Taq* polymerase was unusually low under the amplification conditions employed.

Effect of Individual Loops on Aminoacylation of hs mt tRNA$^{Leu(UUR)}$. All of the selected sequences have numerous substitutions in the D, TΨC, and anticodon loops compared to WT tRNA^{Leu}, making it difficult to deconvolute the role of each loop in aminoacylation. To address the role of each loop individually, we prepared a series of hs mt tRNA$^{Leu(UUR)}$ constructs into which the anticodon, D, and TΨC loops from sequences 21 or 31 were introduced (Figure 5). Sequence 31 exhibits the highest aminoacylation activity and has a D loop sequence similar to that of tRNA$^{Leu(UUR)}$ (only positions 16 and 17 are different), but an anticodon stem featuring three additional base pairs (Figure 5A). To evaluate the effect of the stabilized anticodon stem on aminoacylation, we introduced the anticodon loop of sequence 31 into WT hs mt tRNA$^{Leu(UUR)}$ (construct 31-AC). Sequence 21 has D and TΨC loops that are significantly different from tRNA$^{Leu(UUR)}$, but it retains considerable aminoacylation activity. Four hybrid tRNAs were constructed on the basis of sequence 21 (Figure 5B). Construct 21-AC contains the anticodon loop from sequence 21; constructs 21-D and 21-T feature the D or TΨC loop from sequence 21, respectively, and construct 21-DT features both the D and TΨC loops from sequence 21.

Aminoacylation assays testing the activity of the hybrid constructs are shown in Figure 6. The introduction of the anticodon loop sequences from sequences 21 and 31 into the WT sequence led to a significant increase in aminoacylation compared to WT tRNA$^{Leu(UUR)}$ (Figure 6A). Construct 31-AC, which has three additional Watson−Crick base pairs in the anticodon stem, is better charged than construct 21-AC, which only has a CG base pair and a UG base pair. However, there is no strict correlation between the number of additional base pairs in the anticodon stem and the activity of tRNA in aminoacylation for selected tRNA variants listed in Table 1.

While the stabilized anticodon stems in sequences 21 and 31 increase aminoacylation efficiency, the mutations present in the D and TΨC loops of the variants have a negative effect on aminoacylation. As shown in Figure 6B, constructs 21-D and 21-T each show more than a 15-fold decrease in charging compared to sequence 21 (more than 50-fold decrease when compared to WT tRNA$^{Leu(UUR)}$). However, combining the D and TΨC loop sequences together in construct 21-DT resulted in a partial restoration of charging. This may indicate that the D and TΨC loops in construct 21-DT and sequence 21 interact in a specific manner that stabilizes a folded tRNA structure.

DISCUSSION

Combinatorial approaches have been used to study tRNA−synthetase interactions and to identify or confirm identity elements in several aminoacylation systems (*18, 27, 30−32*). The general idea behind this approach is that if regions randomized in the tRNA library harbor identity elements critical for the recognition by the synthetase, these identity elements should be well-conserved among selected tRNA

sequences. The absence of conservation at particular positions can be interpreted as the absence of specific contact with the synthetase. The in vitro selection strategy described here was designed to monitor the emergence of active sequences from a combinatorial library. Extensive selection would have likely produced sequences that were more efficiently charged than the hs mt tRNA$^{Leu(UUR)}$, as there are several reports of rationally designed sequences with enhanced charging activities (*11*, *12*). Thus, we performed a limited number of selections to obtain a pool of sequences with varied activities so that we might gain insight into features of this tRNA that either promote or attenuate aminoacylation.

Combinatorial Analysis of hs mt tRNA$^{Leu(UUR)}$ Reveals Tolerance to High Variability within the Anticodon Loop. The hs mt tRNA$^{Leu(UUR)}$ variants isolated from the selection displayed essentially no conservation of anticodon loop nucleotides. Changes to the size of the anticodon loop and stem were also tolerated, with many sequences that featured loop nucleotides converted into additional Watson−Crick base pairs exhibiting enhanced aminoacylation (Figure 6A). This observation strongly suggests that there are no sequence-specific contacts between LeuRS and this part of hs mt tRNA$^{Leu(UUR)}$. The results of a previous footprinting study (*11*) are consistent with this idea, as only a weak protection in the anticodon loop was observed in hs mt tRNA$^{Leu(UUR)}$ upon LeuRS binding.

While the anticodon loop does not appear to contain specific nucleotides important for recognition of hs mt tRNA$^{Leu(UUR)}$ by LeuRS, the stability of the anticodon stem does exert a substantial effect on aminoacylation activity. Stabilization of the anticodon stem appears to be the main driving force in the selection of tRNAs from the initial library, as most selected variants share this characteristic. Enhanced aminoacylation activity was observed previously for mutants of hs mt tRNA$^{Leu(UUR)}$ featuring additional or stronger Watson−Crick base pairs within the anticodon stem, and the gain in aminoacylation in both cases was shown to be due to an increase in k_{cat} (*11*, *12*). Chemical and enzymatic probing studies confirm that the anticodon stem is significantly more structured even when just one of the AU base pairs found within the WT structure is simply changed to a GC pair (*12*, *13*). Moreover, probing experiments also revealed that stabilization of the anticodon stem enhances the level of structure of the D stem (*13*). It was therefore proposed that the hs mt tRNA$^{Leu(UUR)}$ has a highly labile structure and unstable domains, and the conformational instability appears amplified because of a high degree of interdomain communication. The results obtained here are consistent with the idea that the hs mt tRNA$^{Leu(UUR)}$ is functionally hindered by its low thermodynamic stability, at least within the context of its reactivity with LeuRS.

The isolation of sequences with extended anticodon stems presents an interesting parallel to the structure of *Staphylococcus aureus* tRNA^{Ile} bound to the cognate IleRS (*33*). This structure revealed the formation of additional base pairs in the anticodon loop that were not predicted from the canonical cloverleaf secondary structure. Thus, the introduction of more base pairs in the hs mt tRNA$^{Leu(UUR)}$ variants may allow the tRNA to preorganize into an active conformation, producing enhanced reactivity.

The in vitro selection system described expedited evolution of sequences active for aminoacylation with no regard to the other functions of tRNAs that could be disrupted. The predominance of sequences that featured enhanced thermodynamic stabilities highlights the importance of a well-folded structure for recognition of hs mt tRNA$^{Leu(UUR)}$ by LeuRS. However, many of the sequence changes observed could perturb other tRNA−protein interactions (e.g. with the ribosome), therefore preventing the adaptation of these advantageous sequence changes in vivo.

Sequence Variations in the D loop of hs mt tRNA$^{Leu(UUR)}$. The combinatorial library of hs mt tRNA$^{Leu(UUR)}$ sequences featured variations mainly at nucleotides not involved in tertiary interactions. It was our intention to preserve the contacts between the D loop and TΨC loop while searching for other nucleotides that might serve as recognition handles for LeuRS. Out of the six randomized positions within the D loop, only two exhibited significant conservation of the nucleotides of the WT sequence: 20a and 21. A21 participates in a triple-base interaction with A14 and U8 in most tRNAs (*25*), thus its conservation within the selected sequences suggests that this same interaction is important in stabilizing the structure of hs mt tRNA$^{Leu(UUR)}$. A20a represents the site of a disease-related mutation (*8*), and the substitution of this A with a G causes a large decrease in aminoacylation (L. Wittenhagen, S. O. Kelley, unpublished data). This adenine could serve as recognition determinant for LeuRS, as it is typically displayed in an accessible conformation in folded tRNA (*25*). Footprinting of hs mt LeuRS on transcript tRNA$^{Leu(UUR)}$ revealed protection from nucleases along the 3′ side of the D loop, with some shifting of the protection pattern towards the 5′ side of the loop observed with native tRNA$^{Leu(UUR)}$ (*11*). This observation, along with the conservation of A20a in our selected sequences, indicates that the D loop of hs mt tRNA$^{Leu(UUR)}$ is an important domain to consider in the search for identity elements within this tRNA.

However, the four other nucleotides that were randomized within the D loop displayed a very high level of variability. Among the selected sequences, the nucleotides found at positions 16, 17, 17a, and 20 rarely corresponded to those found in the WT tRNA$^{Leu(UUR)}$. In particular, only the sequence 25, which was identical to WT, contained the C16. Positions 17 and 17a were frequently uridines, but other bases at these positions produced active sequences. It appears that these nucleotides do not play a large role in modulating aminoacylation of this tRNA by hs mt LeuRS.

Selected hs mt tRNA$^{Leu(UUR)}$ Variants with a Conserved, But Non-Native, TΨC Loop Sequence. All three randomized positions in the TΨC loop of tRNA$^{Leu(UUR)}$ showed remarkable consistency among selected sequences. Interestingly, none retained the WT identity: A57 was replaced by G, and U59 and U60 were both replaced by C. However, when nucleotides G57, C59, and C60 were introduced into hs mt tRNA$^{Leu(UUR)}$ (construct 21-T), this led to a more than 50-fold decrease in aminoacylation rate. This effect is difficult to explain in terms of canonical tRNA structure, since these nucleotides are not typically involved in hydrogen bonding (*25*), and thus replacement of one purine by another, or one pyrimidine by another, should not interfere with tRNA folding.

Interestingly, A57G and U60C are documented pathogenic mutations in hs mt tRNA$^{Leu(UUR)}$ linked to myopathy and MELAS, and a pathogenic mutation resulting in an A57G

substitution has also been found in the hs mt tRNA$^{Leu(CUN)}$ isoacceptor (*8*). Both hs mt tRNA^{Leu} isoacceptors have A57 and U60; thus, it is possible that these nucleotides are identity elements for recognition by the synthetase. The very low level of aminoacylation observed for the construct 21-T supports this idea. The importance of these nucleotides may be masked in other tRNA variants, where stimulated aminoacylation is caused by stabilization of the anticodon stem. It is known that several tRNAs have identity elements in the TΨC loop (e.g. tRNA^{Phe}, tRNA^{Arg} (*34*, *35*)).

The significant effect of nucleotides 57, 59, and 60 on aminoacylation, as observed when the 21-T construct was monitored relative to the WT tRNA$^{Leu(UUR)}$, could also indicate that they are essential for tRNA^{Leu} structure. These nucleotides may provide stabilizing contacts between the D and TΨC loops, though in a way different from canonical tRNAs. This idea is supported by the fact that simultaneous introduction of the non-native D and TΨC loop sequences from variant 21 into hs mt tRNA^{Leu} (construct 21-DT) rescued aminoacylation of both constructs 21-D and 21-T, which suggests an interaction between the D and TΨC loop nucleotides in selected sequences. Generally, the D−TΨC loop interactions in mitochondrial tRNAs are not well understood (*4*), and even though hs mt tRNA$^{Leu(UUR)}$ is believed to have a canonical D−TΨC loop interaction, there may be some additional contacts involving nucleotides that have been randomized in our study.

Cryptic Determinants within the Fragile Structure of hs mt tRNA$^{Leu(UUR)}$. The studies described clearly demonstrate that a stably folded tRNA structure promotes reactivity of hs mt tRNA^{Leu} with LeuRS, but the nucleotides acting as key identity determinants in this complex still remain somewhat cryptic. Our data suggest that the anticodon loop of hs mt tRNA$^{Leu(UUR)}$ does not harbor any identity elements, which is consistent with what has been found for other known leucine aminoacylation systems, with the exception of yeast (*14*−*22*). In other studies of hs mt tRNA$^{Leu(UUR)}$, only A73 and A14 were identified as identity elements (*22*). However, 14 other tRNAs in human mitochondria have A73, and 12 also have the U8-A14 pair (*37*); thus, additional positive determinants are likely to exist in hs mt tRNA^{Leu}, as well as negative determinants against other hs mt tRNAs that have A73. Shape-selective recognition of hs mt tRNA$^{Leu(UUR)}$ should also be considered as a factor contributing to specific aminoacylation of this tRNA by LeuRS, as it seems that mutations are tolerated as long as they provide structural stability. Shape- or structure-based selection of tRNAs may be a more effective means to achieve specificity in human mitochondria than in other systems, given that there is extensive structural heterogeneity in the mt tRNAs. The series of sequences that were selected in the study described here also suggest that the D and TΨC loops contain nucleotides important for efficient aminoacylation of this tRNA by hs mt LeuRS, and further analysis of the structure and function of this tRNA may confirm this proposal.

SUPPORTING INFORMATION AVAILABLE

Gel analyses of selected tRNAs. This material is available free of charge via the Internet at http://pubs.acs.org.

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