Supporting Information

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SI Text

Isolation and Mass Spectrometric Analysis of mt tRNA^{Glu} and mt tRNA^{Gln} from Bovine Liver. Mitochondrial tRNA^{Glu} and tRNA^{Gln} were isolated from fresh bovine liver by acid phenol extraction, anion exchange chromatography, and chaplet column chromatography, as described (1). Purified tRNAs were sequenced by the Donis-Keller method (2) and their posttranscriptional modifications were analyzed by two-dimensional cellulose TLC based on the method of Kuchino et al. (3), as described previously (4). Mass spectrometric analyses of nucleosides and RNA fragments of tRNAs were performed as described (5–8) with minor changes in conditions for the enzymatic reactions. For nucleoside analysis, 4 μg of the individual tRNA was digested by 0.1 units of nuclease P1 (Seikagaku Corporation) and 0.008 units of BAP C75 (Takara Bio Inc.) in 20 μL of 20 mM HEPES-KOH (pH 7.5) for 3 h at 37 °C. To analyze RNA fragments, 2 μg of the individual tRNA was digested using 50 units of RNase T_1 (Epicentre) in 10 μL of 20 mM ammonium acetate (pH 5.3) for 3 h at 37 °C. Conditions and parameters for LC/MS analyses have been described in our previous studies (6, 7).

In Vitro Reconstitution of Gln-tRNA^{Gln} Formation by hGatCAB. [^{14}C]Glu-tRNA^{Glu} and [^{14}C]Glu-tRNA^{Gln} were prepared as described above. After the aminoacylation reactions, the aa-tRNAs were extracted by phenol/chloroform treatment under acidic conditions followed by ethanol precipitation. Residual ATP in the reaction was removed using a gel filtration Nap5 column (GE Healthcare). The transamidation reaction was performed according to (9) with minor modifications. The reactions were conducted at 37 °C for 15 min in 1× AdT buffer [100 mM HEPES-KOH (pH 7.2), 30 mM KCl, 12 mM $MgCl_2$, and 2.5 mM DTT] containing 5 mM ATP, 0.14 mg/mL recombinant hGatCA, 0.06 mg/mL recombinant hGatB, and 65 nM [^{14}C]Glu-tRNA^{Glu} or [^{14}C]Glu-tRNA^{Gln}, in the presence of either 2 mM Gln or 10 mM NH_4Cl, or in the absence of any amide donor. After the reaction, aa-tRNAs were recovered by phenol/chloroform extraction under acidic conditions followed by ethanol precipitation and removal of ATP using a Nap5 column. The amino acids attached to the tRNA were deacylated at 37 °C for 30 min in 0.3% aqueous ammonia. The [^{14}C] labeled amino acids were analyzed by TLC on a cellulose plate (Funakoshi) using a basic solvent system (28% ammonia solution:chloroform:methanol, 1:2:2). The TLC plate was exposed to an imaging plate and visualized using an image analyzer (FLA-7000, Fuji Film).

Construction of Plasmids for mtGluRS and hGatCAB. The cDNAs for mtGluRS, hGatA, hGatB, and hGatC were amplified from total RNA from HeLa cells and cloned into pEGFP-N1 (Clontech) to construct a series of plasmids that express proteins fused to EGFP at their N terminus, namely pEGFP-mtGluRS, pEGFP-hGatA, pEGFP-hGatB, and pEGFP-hGatC. The primers for RT-PCR were as follows: 5′-agtcagtcagtcctcgagatggcg-gcgctcctgag-3′ and 5′-ctgactgactcccgggagctggaaaccaccttctgg-3′ for pEGFP-mtGluRS; 5′-actgactgactcgagatgctgggccg-gagcctccg-3′ and 5′-agtcagtcaggatcctgttttagagagacagaggc-3′ for pEGFP-hGatA; 5′-actgacctcgagatggcggcgcccatgctgcgc-3′ and 5′-agtcagtcaggatccaatgacagcttcttctccag-3′ for pEGFP-hGatB; and 5′-actgagatctatgtggtcgcggttggtg-3′ and 5′-cagtgaattcggctgt-gtgggaatggctc-3′ for pEGFP-hGatC.

For recombinant proteins, the cDNAs for mtGluRS and hGatB lacking the N-terminal mt targeting sequences were cloned into pET-15b (Novagen) and pET-21b (Novagen), respectively. The cDNA for hGatC lacking the N-terminal mt targeting sequence and the full length hGatA were cloned into pET-Duet (Novagen) for co-expression of recombinant proteins. RT-PCR primers used to generate these constructs were as follows: 5′-agtcagtcagtccatatggtgcgagtgcggttcgct-3′ and 5′-actgactgactctcgagctagctggaaaccaccttctg-3′ for mtGluRS; 5′-actgactcatatgctgggccggagcctcc-3′ and 5′-cagtctcgagctgttttagagagacagaggc-3′ for hGatA; 5′-actgacagatctatggcggcgcccatgctgcgc-3′ and 5′-tcagtctcgagcaatgacagcttcttctccag-3′ for hGatB; and 5′-actgactgagtcgaccggatcacggctgcggt-3′ and 5′-agtcagtcagtgcggccgctcagctgtgtgggaatggctc-3′ for hGatC.

Expression and Purification of Recombinant Proteins. *E. coli* rosetta (DE3)pLysS was used as a host strain for the expression of recombinant proteins. The transformants were cultured in LB media containing 100 μg/mL ampicillin and 30 μg/mL chloramphenicol at 37 °C to an OD_{600} value of 0.6. Expression was then induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.1 mM for 20 h at 16 °C. Cells were harvested and suspended in HT buffer [50 mM HEPES-KOH (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, and 7 mM β-mercaptoethanol], and disrupted using sonication at 0 °C. Subsequent purification processes were carried out at 4 °C. Cell lysates were clarified by ultracentrifugation at 100,000 × g for 60 min. The supernatant was loaded onto a nickel-charged HiTrap chelating column (GE Healthcare). After washing out unbound proteins with HT buffer, the recombinant proteins were eluted with a 50-mL linear gradient from 0 to 500 mM imidazole in HT buffer. The fractions containing the recombinant proteins, as judged by SDS/PAGE, were dialyzed against HT buffer overnight. Protein concentrations were determined using a Bio-Rad protein assay kit with BSA as a standard. Glycerol was added to the pooled protein fractions to a final concentration of 10%, and the samples were quick frozen in liquid nitrogen, and stored at −80 °C.

Subcellular Localization of mtGluRS and hGatCAB. To determine the subcellular localization of each EGFP-fused protein, HeLa cells cultured in 35-mm dishes were transfected with 1 μg each of plasmids (pEGFP-mtGluRS, pEGFP-hGatA, pEGFP-hGatB, and pEGFP-hGatC) using FUGENE (Roche Diagnostics). Twenty hours after the transfection, the MitoTracker CMXRos dye (Molecular Probes) was added to a final concentration of 20 nM, and the cells were incubated for 15 min and then washed with fresh DMEM. The green and red fluorescence of EGFP and the MitoTracker dye were observed by confocal microscopy LEICA DM IRBE (Leica).

RNA Interference. To investigate the function of hGatCAB in mammalian mitochondria, each subunit of hGatCAB was knocked down by RNAi. siRNAs for hGatA, hGatB and hGatC were designed using the siRNA design algorithm "siExplorer" (10), and synthesized as described previously (11). The sense strand sequence of each siRNA is as follows: 5′-cagcgagaaugaa-gauuca-3′ for hGatA; 5′-cuaugaaauucagaggcaa-3′ for hGatB; and 5′-gguaauaucucuuugccaa-3′ for hGatC. An siRNA for luciferase (5′-cguacgcggaauacuucga-3′) was used as a control. HeLa cells (7.5×10^5 cells) were plated in 100-mm dishes and cultured for 24 h. The medium was then replaced with 10 mL OPTI-MEM I (GIBCO). The cells were transfected with 100 pmol of the siRNA (final concentration, 10 nM) using 20 μL

Oligofectamine (Invitrogen) for 5 h, after which 5 mL DMEM containing 30% FBS was added. At 96 h post-transfection, total RNA was extracted from the cells using the TRI Reagent (Sigma), according to the manufacturer's instructions. Quantitative RT-PCR (12) using a LightCycler480 (Roche Diagnostics) was performed to measure the level of each mRNA using the following sets of primers:

5′-actgtgggcttgttggtttc-3′ and 5′-aaactgggaagcatgaatgg-3′ for hGatA; 5′-ggctaccaaattacccagca-3′ and 5′-cactcctgccctgttcaaat-3′ for hGatB; 5′-cgtctagcgcttgtggactt-3′ and 5′-cgcgatgggagttttgtagt-3′ for hGatC; and 5′-gtcttcaccaccatggagaagg-3′ and 5′-atgatcttgaggctgttgtcat-3′ for GAPDH.

Aminoacylation Assays. The aminoacylation reactions were carried out at 37 °C with reaction mixtures (60 μL) containing 50 mM HEPES-KOH (pH 7.6), 20 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 1 mM spermine, 20 μM [^{14}C]L-glutamate (9.36 GBq/mmol), 0.01 A_{260} unit of tRNA, and 0.1 μg/μL mtGluRS. The initial rates of aminoacylation were ascertained using different tRNA concentrations ranging from 0.1 to 1.2 μM. Samples taken at various time points were spotted onto Whatman 3MM filter disks. The disks were washed with 5% trichloroacetic acid, and the radioactivity was measured by liquid scintillation counting. To obtain reasonable kinetic plots for determining the apparent K_m and k_{cat} values, we used the optimal concentrations of mtGluRS, 7.6 nM for mt tRNA^{Glu} and 15.2 nM for mt tRNA^{Gln}.

Measuring Cell Proliferation in Non-Fermentable Growth Conditions. HeLa cells (7.5×10^5 cells) were cultured and transfected with an siRNA for hGatA, hGatB, or hGatC (final concentration, 10 nM), as described above. At 24 h post-transfection, the cells in each dish were washed with PBS and trypsinized, collected, and 4×10^3 cells were subcultured in 96-well plates for 6 days either in "glucose medium," containing 1 mg/mL glucose, or in "galactose medium," containing 1 mg/mL galactose. Cell proliferation in each well was evaluated daily using alamarBlue™ (Biosource International, Inc.) according to the manufacturer's instructions. The absorbance of the wells was read at 570 nm and 600 nm with a Spectramax 190 plate reader (Molecular Devices, Inc.).

Status of Aminoacylation in Cells, Measured by Northern Blotting. HeLa cells (7.5×10^5 cells) were cultured and transfected with siRNA for hGatA (final concentration, 10 nM) or a mixture of the three siRNAs for all subunits of hGatCAB (final concentration, 3.3 nM each), as described above. At 72 h post-transfection, total RNA was extracted from the cells using the TRI Reagent (Sigma) according to the manufacturer's instructions. Total RNA (6 μg) was electrophoresed in an acidic polyacrylamide gel at 4 °C, as described previously (13), and blotted onto a Hybond-*N* + membrane (GE Healthcare). Mitochondrial tRNA^{Gln} and tRNA^{Glu} were detected with 5′-^{32}P-labeled oligonucleotide probes whose sequences were 5′-ctaggactatgagaatcgaacccatccctg-3′ for mt tRNA^{Gln} and 5′-tattctcgcacggactacaaccacgaccaa-3′ for mt tRNA^{Glu}. The membrane was exposed to an imaging plate, followed by analysis with an image analyzer (FLA-7000, Fuji Film).

Complex Formation of hGatCAB Monitored by Gel Filtration Chromatography. To investigate the molecular interaction between hGatCA and hGatB, gel filtration chromatography was performed using Superdex 200 HR10/30 (GE Healthcare) with a mobile phase consisting of 50 mM Tris-KOH (pH 7.6) and 200 mM KCl. The recombinant hGatCA (0.7 mg/mL) and hGatB (0.5 mg/mL) were first mixed in a buffer consisting of 100 mM HEPES-KOH (pH 7.2), 30 mM KCl, 12 mM $MgCl_2$, and 2.5 mM DTT, incubated at 37 °C for 10 min, and 100 μL samples were then loaded onto the column. Chromatography was monitored by UV absorption at 220 nm. Each fraction was precipitated with trichloroacetic acid, washed with acetone, and an aliquot was then resolved by SDS/PAGE using a 15–25% gradient gel (Daiichi Pure Chemicals). The samples were analyzed by Western blotting with anti-polyhistidine (Sigma) to detect hGatB.

Hydrolysis Protection Assay. The assay was performed according to (14). Briefly, recombinant mtEF-Tu was overexpressed using a plasmid kindly provided by Dr. Spremulli (University of North Carolina). [^{3}H]Glu-tRNA^{Glu} and [^{3}H]Glu-tRNA^{Gln} were prepared as described above. [^{3}H]Glu-tRNA^{Gln} was then converted to [^{3}H]Gln-tRNA^{Gln} using the transamidation reaction described above. The recombinant mtEF-Tu was preincubated at 30 °C for 10 min in a reaction mixture consisting of 75 mM Tris-HCl (pH 7.5), 75 mM NH_4Cl, 15 mM $MgCl_2$, 7.5 mM DTT, 60 μg/mL BSA, 0.1 mM GTP, 2.25 mM phosphoenolpyruvate, and 2.5 units/ml pyruvate kinase. Either [^{3}H]Glu-tRNA^{Glu}, [^{3}H]Glu-tRNA^{Gln}, or [^{3}H]Gln-tRNA^{Gln} was then added to the mixture. The deacylation reaction was performed at 30 °C. Samples taken at various time points were spotted onto Whatman 3MM filter disks. The disks were washed with 5% trichloroacetic acid, and the radioactivity was measured by liquid scintillation counting.

Isolation of Human Cytoplasmic tRNA^{Gln}. Human cyto tRNA^{Gln} was isolated from tRNA fractions prepared from human placenta as described (15) by the chaplet column chromatography (1) using a synthetic DNA probe with 3′ biotin that is complementary to cyto tRNA^{Gln} (5′-tggaggttccaccgagatttgaactcggatcgctg-3′). The isolated cyto tRNA^{Gln} was incubated at 65 °C for 10 min and annealed at room temperature in a buffer containing of 50 mM HEPES-KOH (pH 7.5) and 10 mM $MgCl_2$. The tRNA was treated with T4 polynucleotide kinase (0.3 U/μL, Toyobo) at 37 °C for 1.5 h to dephosphorylate its 3′ end. Then, the 3′ end was repaired with *E. coli* CCA-adding enzyme as described (16). The repaired cyto tRNA^{Gln} was finally purified by 10% PAGE in a 40-cm gel containing 7 M urea. Six micrograms of the purified cyto tRNA^{Gln} was obtained. The purity of the isolated tRNA was estimated by analyzing RNA fragments by mass spectrometry as described (6, 7).

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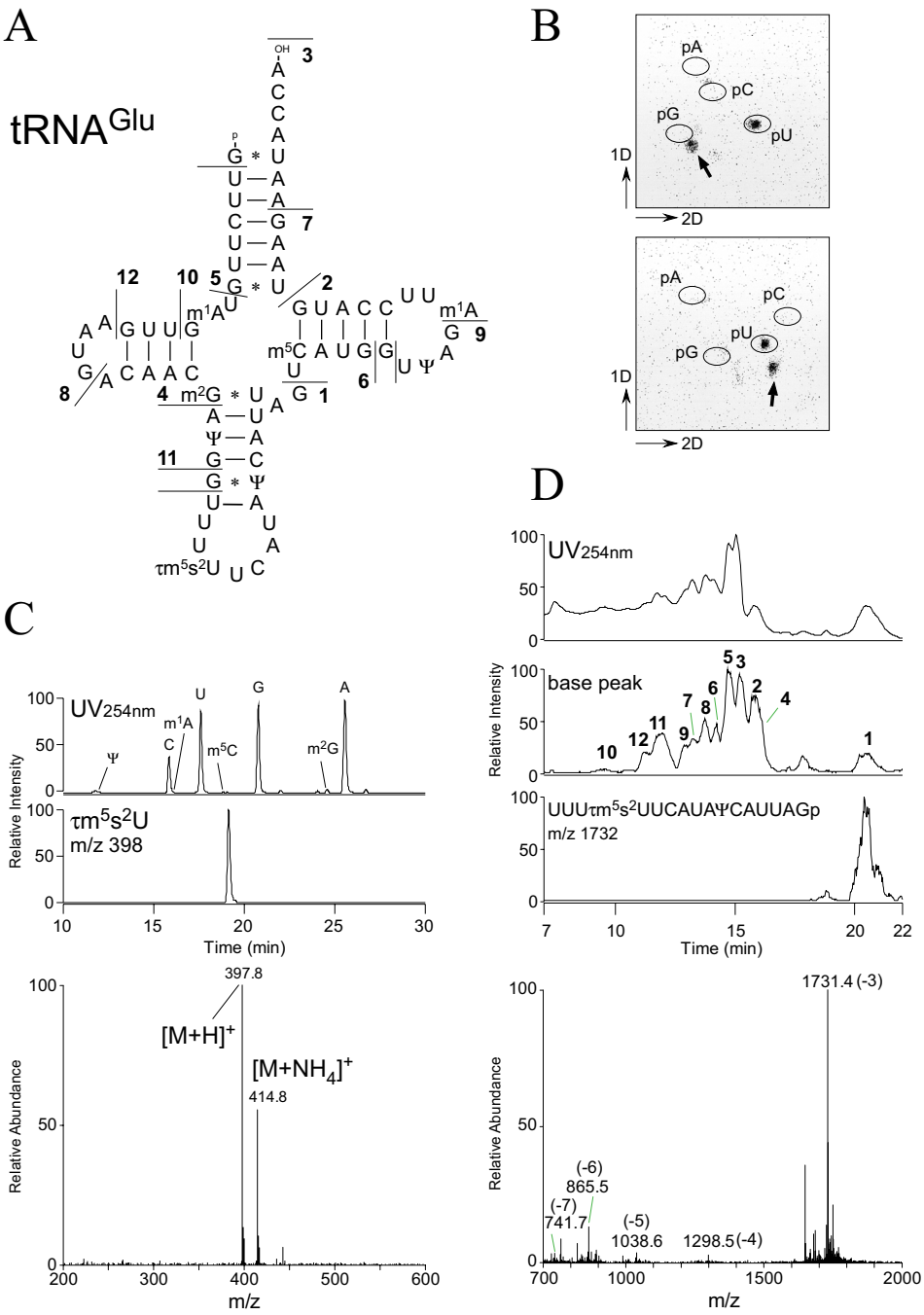

Fig. S1. Mass spectrometry analysis of modified nucleosides in bovine mt tRNA^{Glu} and mt tRNA^{Gln}. (*A* and *E*) Secondary structures of the mt tRNA^{Glu} (*A*) and mt tRNA^{Gln} (*E*) showing the RNase T1 cleavage sites. Numbers of the RNA fragments are the same as in Tables S1 and S2. The complete sequence of tRNA^{Glu} has been submitted as tdbR00000053 in the tRNAdb database (http://trnadb.bioinf.uni-leipzig.de/) (17). (*B* and *F*) Two-dimensional, TLC analyses of the wobble modifications of mt tRNA^{Glu} (*B*) and mt tRNA^{Gln} (*F*) using the method of Kuchino (3). 5′-^{32}P labeled nucleosides at the wobble positions were analyzed using a two-solvent system as follows. System A (upper panel), 1st dimension, isobutyric acid:NH_4OH:H_2O (66:1:33), and 2nd dimension, isopropyl alcohol:HCl:H_2O (70:15:15). System B (lower panel), 1st dimension, as in System A, and 2nd dimension, 0.1 M sodium phosphate (pH 6.8) (100 ml) + ammonium sulfate (60 g) + n-propanol (2 mL). Open ellipses indicate the positions of unmodified 5′-mononucleotides (pA, pG, pC, and pU). The pτm^{5}s^{2}U spots are indicated by arrows. (*C* and *G*) LC/MS nucleoside analyses of mt tRNA^{Glu} (*C*) and mt tRNA^{Gln} (*G*). The top panels show chromatograms of UV absorption at 254 nm and the identities of the nucleosides are indicated. The middle panels show mass chromatogram detection of τm^{5}s^{2}U with *m/z* 398. The bottom panels show mass spectra for τm^{5}s^{2}U. The positive ions of the proton adduct (*m/z* 398) and ammonium adduct (*m/z* 415) are indicated. (*D* and *H*) LC/MS analyses of RNA fragments digested by RNase T_1 for mt tRNA^{Glu} (*D*) and mt tRNA^{Gln} (*H*). The top panels show chromatograms of UV absorption at 254 nm. The second panels show base-peak mass chromatograms and the identities of the RNA fragments are indicated. The numbers of the fragments are the same as in Fig. S1 *A* and *E*, and Tables S1 and S2. The third panels show mass chromatograms of the triply-charged (*m/z* 1731.4) and doubly-charged (*m/z* 869.4) ions of the anticodon-containing fragments from mt tRNA^{Glu} and mt tRNA^{Gln}, respectively. The bottom panels show the mass spectra of the anticodon-containing fragments.

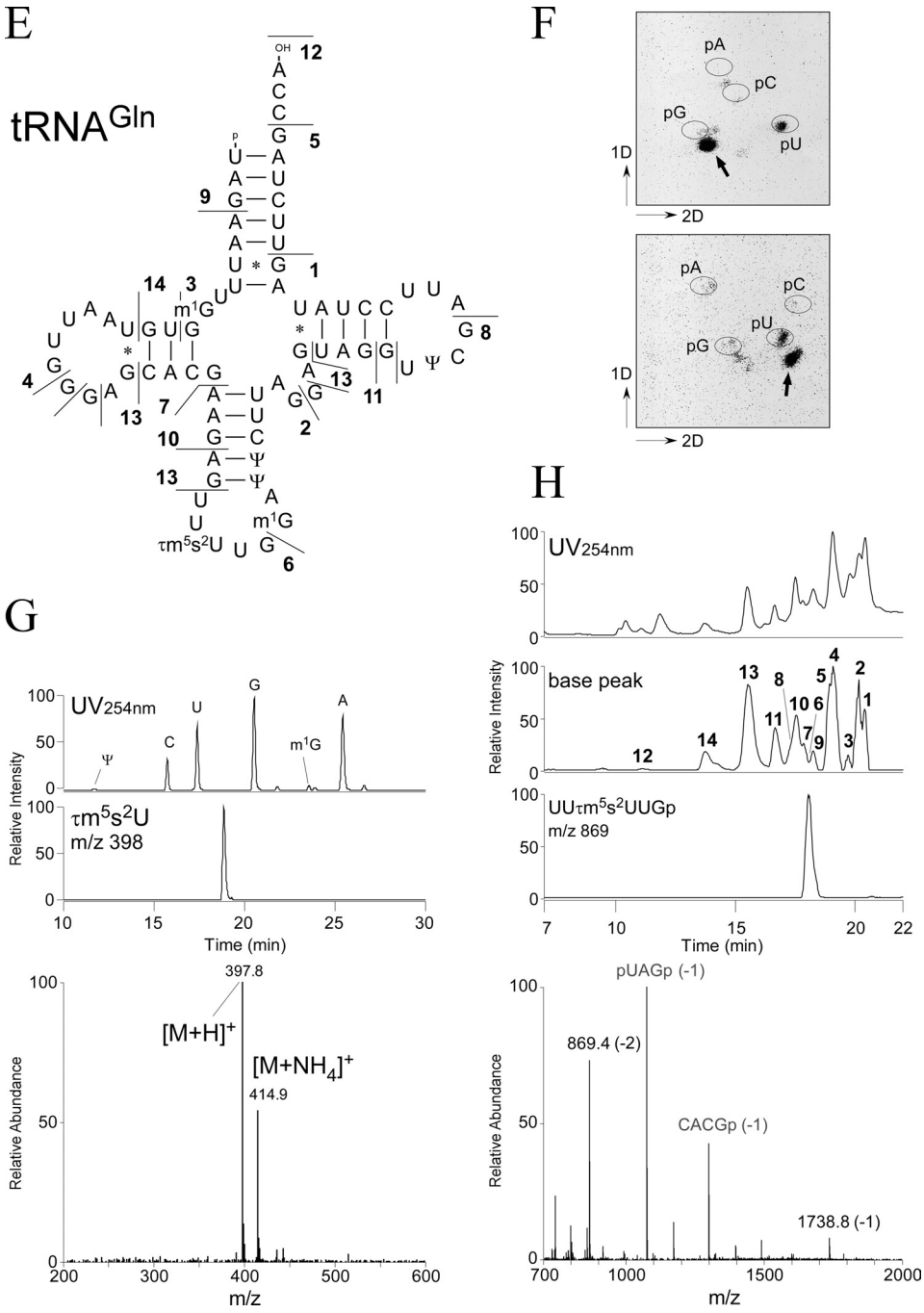

Fig. S1 continued.

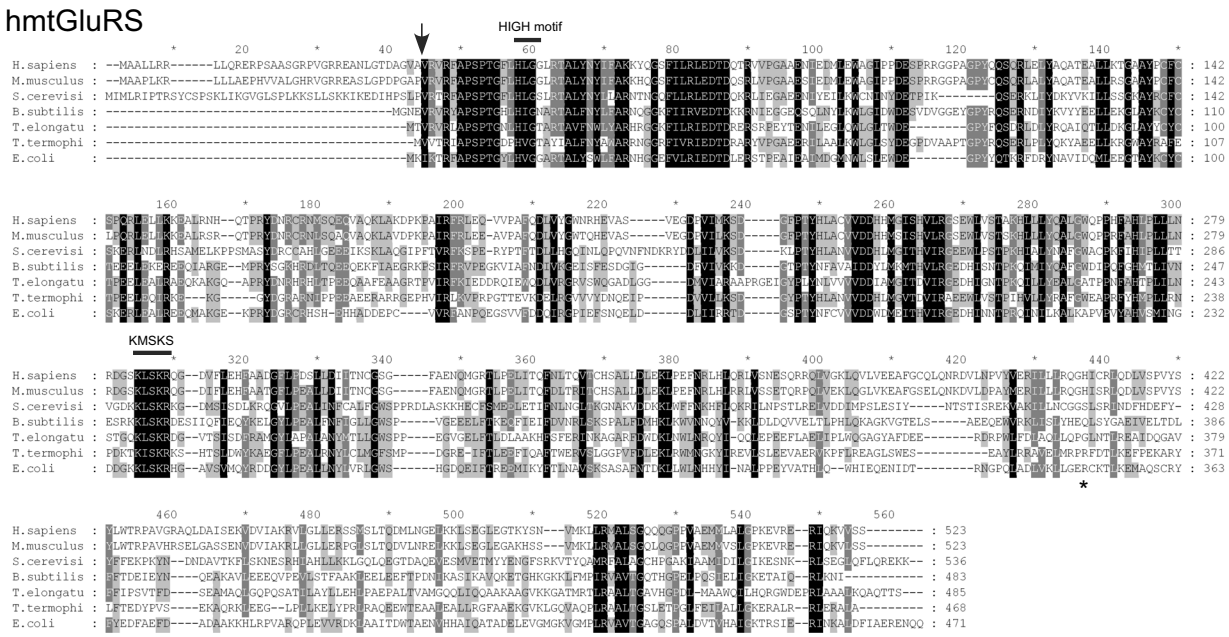

Fig. S2. Sequence alignment of human mtGluRS with other mitochondrial and bacterial GluRS. Amino acid sequence alignment of mtGluRS proteins from human (*H. sapiens*), mouse (*M. musculus*), and yeast (*S. cerevisiae*), with bacterial GluRS proteins from *B. subtilis*, *T. elongatus*, *T. thermophilus*, and *E. coli*. The predicted cleavage site of the N-terminal mitochondrial targeting sequence is indicated by the arrow. Signature motifs of class I aaRS (HIGH and KMSKS) are indicated. The black and gray boxes indicate regions of sequence identity (black) and sequence similarity (gray). The position corresponding to Arg-358 in *T. thermophilus* GluRS is indicated by an asterisk.

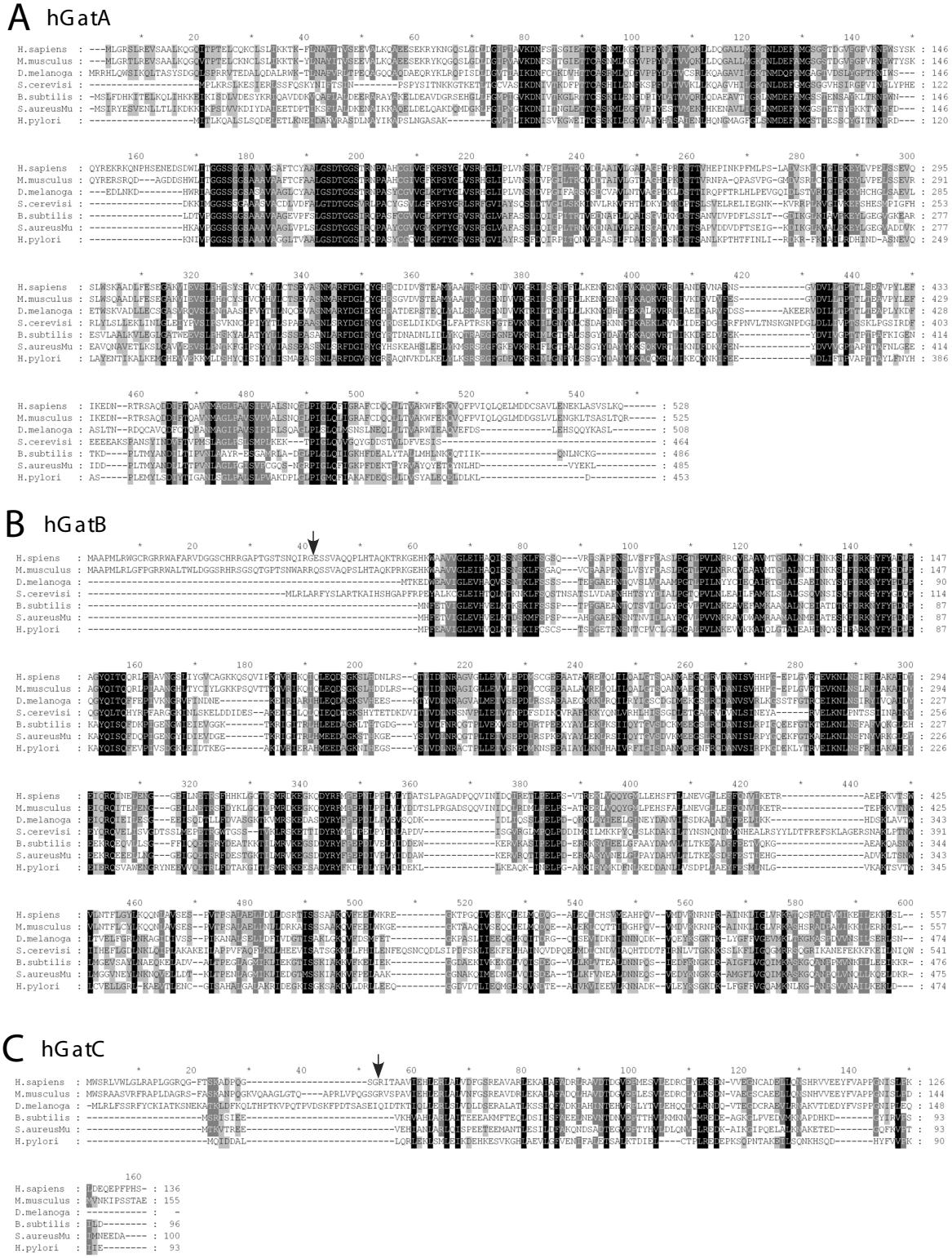

Fig. S3. Sequence alignments of human GatA (*A*), GatB (*B*), and GatC (*C*) with other mitochondrial and bacterial homologs. Amino acid sequence alignment of each subunit of GatCAB from human (*H. sapiens*), mouse (*M. musculus*), *Drosophila* (*D. melanogaster*), yeast (*S. cerevisiae*), *B. subtilis*, *S. aureus*, and *H. pylori*. The predicted cleavage site of the N-terminal mitochondrial targeting sequence of each subunit is indicated by an arrow. The black and gray boxes indicate regions of sequence identity (black) and sequence similarity (gray).

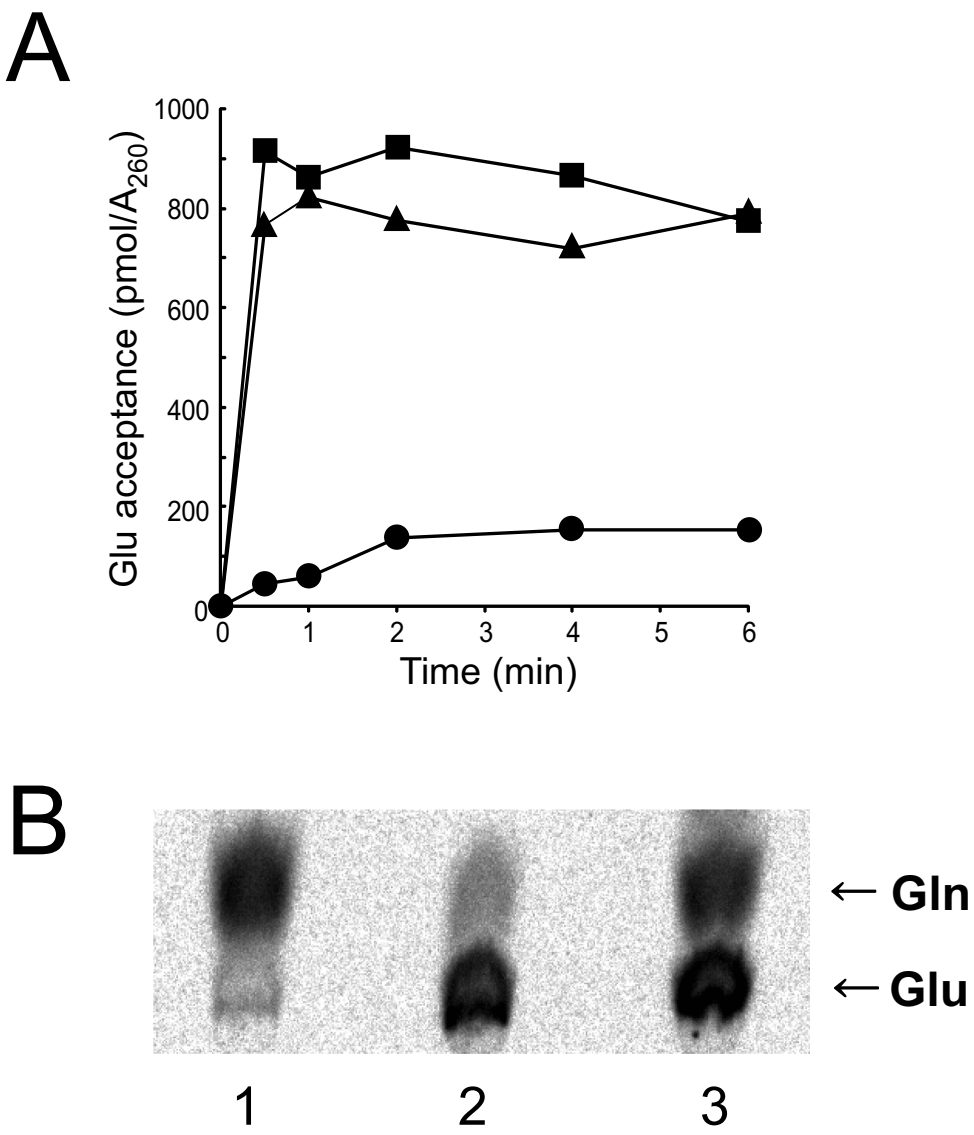

Fig. S4. Cytoplasmic $tRNA^{Gln}$ is a substrate for mtGluRS and hGatCAB. (*A*) Glutamate-charging activities of mt $tRNA^{Glu}$ (square), mt $tRNA^{Gln}$ (triangle), and cyto $tRNA^{Gln}$ (circle) by recombinant mtGluRS. (*B*) In vitro reconstitution of Gln-cyto $tRNA^{Gln}$ formation by the recombinant hGatCAB. [^{14}C]-labeled Glu-mt $tRNA^{Gln}$ (lane 1) or Glu-cyto $tRNA^{Gln}$ (lane 2) was used as a substrate for transamidation by hGarCAB. Positions of Gln and Glu on TLC were determined using [^{14}C]Gln and [^{14}C]Glu as markers (lane 3).

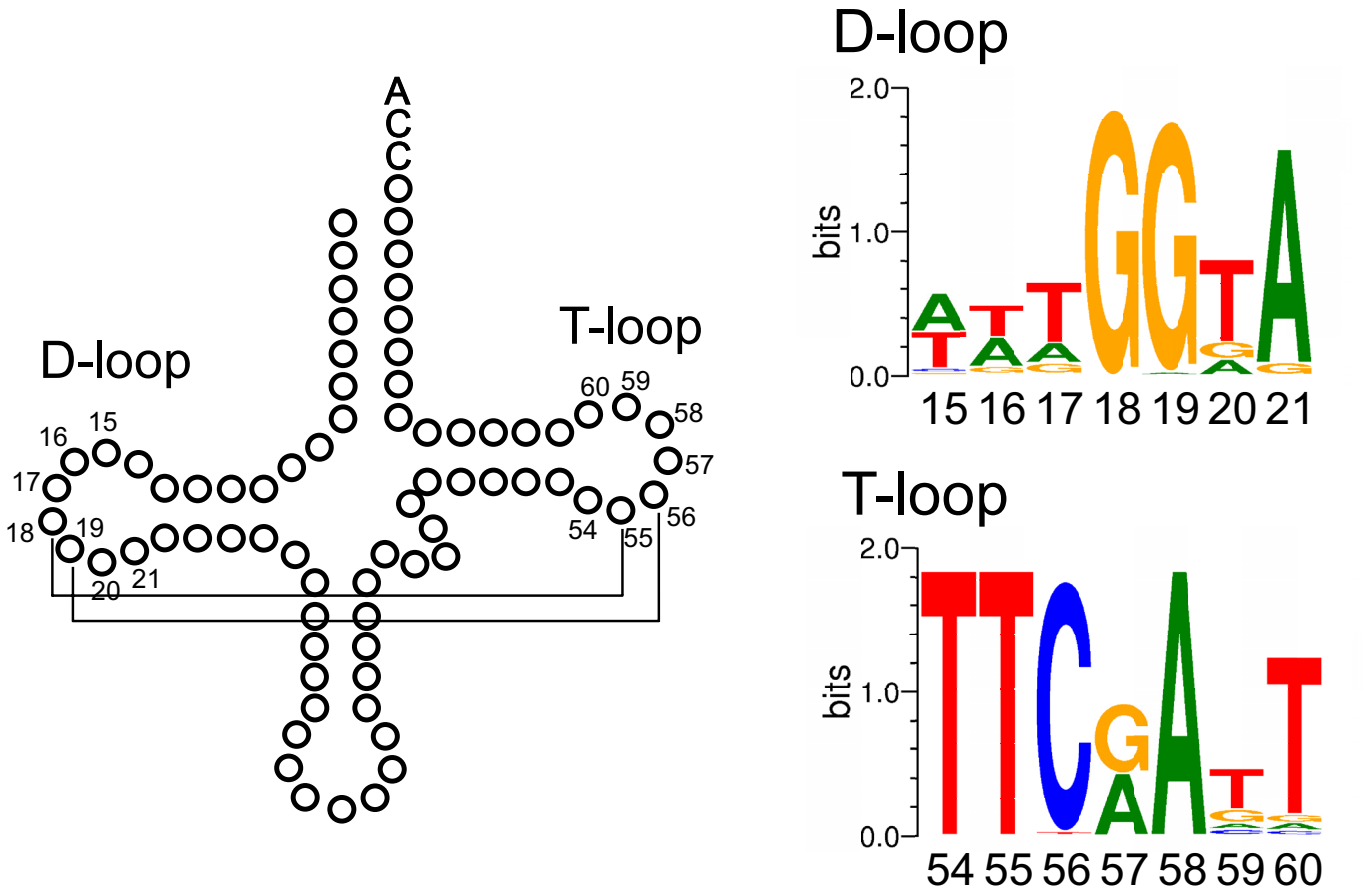

Fig. S5. Phylogenetic conservation of D- and T-loop sequences of mammalian mt $tRNA^{Gln}$. Cloverleaf schematic of tRNA with bases in the D- and T-loops numbered. Tertiary interactions between positions 18/19 and 55/56 are connected. D- and T-loop sequences of 145 mammalian mitochondrial $tRNA^{Gln}$ obtained from the Mamit-tRNA database (http://mamit-tRNA.u-strasbg.fr) (18) are aligned (right panels). Phylogenetic conservation of each base in D- and T-loops was calculated as described by WebLogo (19, 20).

Table S1. RNase T1 fragments from *Bovine* mitochondrial $tRNA^{Glu}$ detected by MS analysis

No.	Sequences of RNA fragments	Molecular mass	Mono-isotopic m/z		Charge state
			Observed	Calculated	
1	UUUτm^5s^2UUCAUAΨCAUUAGp	5196.6	1731.4	1731.2	−3
2	m^1AUUCCAUGp	2563.3	1280.9	1280.7	−2
3	AAUACCA$_{(OH)}$ (3′ terminus)	2170.4	1084.4	1084.2	−2
4	ACAACm^2Gp	1974.3	986.4	986.1	−2
5	UUCUUGp	1892.2	945.4	945.1	−2
6	Um^5CAUGp	1623.2	810.8	810.6	−2
7	UAAGp	1327.2	1326.1	1326.2	−1
8	AAUGp	1327.2	1326.1	1326.2	−1
9	UΨAGp	1304.2	1303.0	1303.2	−1
10	Um^1AGp	1012.2	1011.2	1011.1	−1
11	AΨGp	998.1	997.1	997.1	−1
12	UUGp	975.1	974.0	974.1	−1

Table S2. RNase T1 fragments from *Bovine* mitochondrial tRNA^{Gln} detected by MS analysis

No.	Sequences of RNA fragments	Molecular mass	Mono-isotopic m/z		Charge State
			Observed	Calculated	
1	AUUCCUAUAGp	3184.4	1591.5	1591.2	−2
2	m^1GAΨΨCUUAGp	2909.4	1454.2	1453.7	−2
3	AAUUUm^1GGp	2298.3	1148.5	1148.1	−2
4	UAAUUGp	1939.2	968.6	969.2	−2
5	UUCUAGp	1915.2	956.6	957.4	−2
6	UU$_\tau$ m^5s^2UUGp	1740.2	869.4	869.1	−2
7	CACGp	1302.12	1301.3	1301.2	−1
8	UΨCGp	1280.1	1279.1	1279.1	−1
9	pUAGp (5′ terminus)	1078.1	1077.2	1077.1	−1
10	AAGp	1021.2	1020.3	1020.2	−1
11	UAGp	998.1	997.1	997.1	−1
12	$CCA_{(OH)}$ (3′ terminus)	877.2	876.2	876.2	−1
13	AGp	692.1	691.2	691.1	−1
14	UGp	669.1	668.1	668.1	−1

Table S3. Kinetic parameters of glutamylation of bovine mt $tRNA^{Glu}$ and mt $tRNA^{Gln}$

	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m, $\mu M^{-1}\ s^{-1}$
$tRNA^{Glu}$	0.27 (0.265, 0.274)	0.31 (0.314, 0.304)	1.15
$tRNA^{Gln}$	1.7 (1.59, 1.80)	0.95 (0.942, 0.962)	0.56

These parameters are estimated values determined in the range of 0.1–1.2 μM tRNA concentration. Each parameter was determined by averaging values (in parentheses) obtained from two independent experiments.