In vivo identification of essential nucleotides in tRNA\textsuperscript{Leu} to its functions by using a constructed yeast tRNA\textsuperscript{Leu} knockout strain

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ABSTRACT

The fidelity of protein biosynthesis requires the aminoacylation of tRNA with its cognate amino acid catalyzed by aminoacyl-tRNA synthetase with high levels of accuracy and efficiency. Crucial bases in tRNA\textsuperscript{Leu} to aminoacylation or editing functions of leucyl-tRNA synthetase have been extensively studied mainly by in vitro methods. In the present study, we constructed two Saccharomyces cerevisiae tRNA\textsuperscript{Leu} knockout strains carrying deletions of the genes for tRNA\textsuperscript{Leu}(GAG) and tRNA\textsuperscript{Leu}(UAG). Disrupting the single gene encoding tRNA\textsuperscript{Leu}(GAG) had no phenotypic consequence when compared to the wild-type strain. While disrupting the three genes for tRNA\textsuperscript{Leu}(UAG) had a lethal effect on the yeast strain, indicating that tRNA\textsuperscript{Leu}(UAG) decoding capacity could not be compensated by another tRNA\textsuperscript{Leu} isoacceptor. Using the triple tRNA knockout strain and a randomly mutated library of tRNA\textsuperscript{Leu}(UAG), a selection to identify critical tRNA\textsuperscript{Leu} elements was performed. In this way, mutations inducing in vivo decreases of tRNA levels or aminoacylation or editing ability by leucyl-tRNA synthetase were identified. Overall, the data showed that the triple tRNA knockout strain is a suitable tool for in vivo studies and identification of essential nucleotides of the tRNA.

INTRODUCTION

Aminoacyl-tRNA synthetases (aaRSs) are a family of key enzymes present in three kingdoms of life (1). They catalyze aminoacylation of their cognate tRNAs with amino acids to form correct aminoacyl-tRNAs, which are the substrates of protein biosynthesis in the ribosome. Generally, aminoacylation of tRNA is performed by a two-step reaction—activation of the amino acid with ATP and its subsequent transfer to the 3’-end of the tRNA (2). Based on structural features of the amino acid activation domain, aaRSs can be divided into two classes—I and II (3). The fidelity of the aminoacylation reaction for certain aaRSs is threatened by a limited number of non-cognate standard amino acids. However, some aaRSs have evolved proofreading mechanisms to hydrolyze misactivated amino acids or misacylated tRNAs, known as pre-transfer editing or post-transfer editing, respectively (4).

Leucyl-tRNA synthetase (LeuRS) belongs to class Ia aaRS, which is characterized by a Rossmann fold with two signature peptides, HIGH and KMSKS. Additionally, almost all LeuRS contain a large insertion domain called connective peptide 1 (CP1) within the catalytic Rossmann fold domain. CP1 is defined as a classic editing domain, which can hydrolyze the aminoacyl bond of mischarged aa-tRNA to ensure the fidelity of the catalytic process (5,6).

The cognate substrate of LeuRS, tRNA\textsuperscript{Leu}, has multiple unique characteristics. First, it belongs to class II tRNAs, along with tRNA\textsuperscript{Ser} and tRNA\textsuperscript{Tyr}, characterized by a long variable stem and loop while other tRNAs only harbor a short variable arm (7). Second, in the normal genetic code, leucine is specified by six different codons and at least four tRNA\textsuperscript{Leu} isoacceptors exist in vivo. Therefore, how LeuRS recognizes the isoacceptors exhibiting diverse sequences and structural heterogeneity remains an interesting question. Third, LeuRS contains both an amino acid activation domain and an editing domain. The 3’-end of tRNA\textsuperscript{Leu} should shuttle between the two active sites during aminoacylation and editing processes. Our recent studies have shown that translocation of the tRNA\textsuperscript{Leu} 3’-terminus is crucial for the enzymatic activity (8).
Our laboratory has investigated the interaction between LeuRS and \textit{tRNA}^{\text{Leu}} from various species, including \textit{Escherichia coli}, \textit{Aquifex aeolicus}, \textit{Giardia lamblia} and human (cytosolic and mitochondrial) (6,8–11). The above-mentioned \textit{tRNA}^{\text{Leu}} could be purified after overexpression in \textit{E. coli} or transcribed by T7 RNA polymerase \textit{in vitro}. Several extensive \textit{in vitro} studies have been carried out to clarify the recognition elements of \textit{tRNA}^{\text{Leu}} from different species during the LeuRS-t\textit{tRNA}^{\text{Leu}} interaction. A73, which is absolutely conserved in all \textit{tRNA}^{\text{Leu}}, was found to be the discriminator base. In \textit{E. coli}, the amino acid-accepting end (CCA	extsubscript{32}) has been shown to be essential for both aminoacylation and editing (8). Similarly, the \textit{tRNA} folding resulting from the tertiary interactions between the D-loop and the TΨC-loop is crucial for recognition by LeuRS during both steps (9).

In the bacterium \textit{A. aeolicus}, interactions between LeuRS and \textit{tRNA}^{\text{Leu}} anticodon arm are essential for translocation of the \textit{tRNA} from the synthetic to the editing site (10,12). However, nucleotides of \textit{tRNA}^{\text{Leu}} that specifically control the editing reaction are unknown. In \textit{tRNA}^{\text{Leu}}, the specific nucleotides from the D-loop are sufficient to trigger the editing response (13). In \textit{tRNA}^{\text{Leu}} both aminoacylation and editing have been shown to be sensitive to the G3•U70 base pair in the acceptor stem (14).

Despite considerable efforts, transcripts of \textit{tRNA}^{\text{Leu}} from \textit{Saccharomyces cerevisiae} (yeast \textit{tRNA}^{\text{Leu}}) could not be synthesized \textit{in vitro} with a reasonable accepting activity (15). \textit{In vitro} transcription by T7 RNA polymerase is a powerful approach to study recognition elements of \textit{tRNAs}; but it has some limitations. First, the point-by-point site-directed mutagenesis method is time-consuming if an exhaustive investigation is carried out. Second, \textit{in vitro} transcribed \textit{tRNAs} may be inactive due to the absence of critical base modifications (16). An alternative approach consists in using \textit{in vivo} assays, which may overcome some of these limitations and approach the physiological conditions. Thus, in some conditions, it may become a useful substitute for the T7 RNA transcription approach. \textit{In vivo} screening assays are often based on \textit{tRNA} knockout strains. In \textit{E. coli}, new approaches such as one-step inactivation of chromosomal genes and specific plasmids have been developed to delete a \textit{tRNA} gene in order to investigate its structure and function \textit{in vivo} (17,18). In eukaryotes, a yeast \textit{tRNA} knockout strain was used to discriminate between active and inactive \textit{tRNA} molecules (19). In mammals, a \textit{tRNA} knockout mouse was constructed, with the aim of studying the relationship between organic aging and selenocysteine metabolism (20,21).

Here, we constructed two knockout yeast strains for \textit{tRNA}^{\text{Leu}} genes. In the first strain, the unique gene of \textit{tRNA}^{\text{Leu}}(GAG) was deleted, and the three genes of \textit{tRNA}^{\text{Leu}}(UAG) were deleted in the second. These strains were used to study the two \textit{tRNAs} \textit{in vivo}. The results showed that \textit{tRNA}^{\text{Leu}}(UAG), but not \textit{tRNA}^{\text{Leu}}(GAG), is essential for yeast growth. A genetic selection was performed to isolate lethal mutants of \textit{tRNA}^{\text{Leu}}(UAG) unable to complement the yeast knockout strain. In addition, selection on plates containing the toxic analog norvaline (Nva) led to the identification of \textit{tRNA} mutants unable to be edited by LeuRS (4,5,22). In this way, seven critical nucleotides for aminoacylation of yeast \textit{tRNA}^{\text{Leu}}(UAG) and one for the editing function of yeast LeuRS were identified.

**MATERIALS AND METHODS**

**Materials**

Ethylendiaminetetraacetic (EDTA), PEG4000, lithium acetate, polyvinylpyrrolidone (PVP), bovine serum albumin (BSA), Ficoll 400, sodium citrate, potassium acetate, glucose, magnesium chloride, Tris–HCl and maleic acid were purchased from Sango (Shanghai, China). The yeast minimal synthetic defined (SD) base and dropout (DO) supplement base were purchased from Clontech (Mountain View, CA, USA). 5-fluoroorotic acid (5-FOA), restriction endonucleases and T4 DNA ligase were obtained from Fermentas (Burlington, Canada). Amino acids were purchased from Sigma (St Louis, MO, USA). The KOD plus DNA polymerase and dNTPs were purchased from TOYOBO (Osaka, Japan). Digoxigenin blocking reagent and anti-digoxigenin-AP were purchased from Roche (Basel, Switzerland). All the radioactive amino-acids and ATP are purchased from PerkinElmer (Waltham, MA, USA). T7 RNA polymerase was prepared in our laboratory as previously described. (23). Streptavidin Agarose Resin was purchased from Thermo-Pierce (Rockford, IL, USA). Ni	extsuperscript{2+}–NTA superflow column was purchased from Qiagen (Düsseldorf, Germany).

Plasmids pALR5 (\textit{ir4}, \textit{URA}, \textit{ADE3} (24), pRS313 (\textit{His3}, \textit{Cen6}, \textit{Arsh4}), pRS314 (\textit{Trp1}, \textit{Cen6}, \textit{Arsh4}), pRS315 (\textit{Leu2}, \textit{Cen6}, \textit{Arsh4}) and pGPD415 (\textit{Leu2}, \textit{Cen6}, \textit{Arsh4}, GPD promoter) (25,26) were from the Institut de Biologie Moléculaire et Cellulaire du CNRS (Strasbourg, France); pET28-\textit{ycleuS} (\textit{Kan}, \textit{ycleuS}) and pET28-\textit{ycleuS}-\textit{D419A} (\textit{KAN}, \textit{ycleuS}-\textit{D419A}) were constructed previously and stored in our laboratory (22). \textit{E. coli} strain BL21(DE3) was purchased from Stratagene (La Jolla, CA, USA). pRS317 (\textit{Lys2}, \textit{Cen6}, \textit{Arsh4}) and pRS412 (\textit{Ade2}, \textit{Cen6}, \textit{Arsh4}), \textit{S. cerevisiae} strains YPH499 (\textit{Mata ure3-52 lys2-801\textsuperscript{Amer} ade2-104\textsuperscript{Ochre} trp1-\textit{A63 his3-\textit{A200 leu2-11}}) and YPH501 (\textit{ura3-52 lys2-801\textsuperscript{Amer} ade2-104\textsuperscript{Ochre} trp1-\textit{A63 his3-\textit{A200 leu2-11}}) (26,27) were gifts from JinQiu Zhou in our institute. Digoxigenin-labeled RNA probes were synthesized by Takara (Otsu, Shiga, Japan).

**Gene cloning and site-directed mutagenesis**

The yeast \textit{tRNA}^{\text{Leu}}(UAG) gene and its upstream and downstream DNA fragments were amplified from genome extracted from wild-type (WT) yeast strain YPH501 by PCR using forward primer 5\textsuperscript{\prime}–\textit{AAAGAGCT CGACAAACGACTG}–3\textsuperscript{\prime} (SacI site in italics) and reverse primer 5\textsuperscript{\prime}–\textit{AAAGATCCCGTGGCGCCAAGA}–3\textsuperscript{\prime} (BamHI site in italics), which introduced restriction sites for cloning. After digestion with SacI and BamHI, the PCR product was inserted into the same sites of pALR5 to give the rescuing plasmids pALR-T [\textit{ir4}, \textit{URA}, \textit{ADE3}, \textit{TL}/(UAG)]. Similarly, the digested PCR product was
inserted into pRS314 to give pRS314-R [TRPI, CEN6, ARSH4, TL(UAG)] which was used as a template for the random mutagenesis. The single-point mutants of tRNA^{Leu}(GAG) were constructed by PCR according to the protocol provided by the KOD-Plus-Mutagenesis Kit (TOYOBO, Japan) (28), using mutation-containing primers and pRS314-R as template.

An additional plasmid expressing a chimeric tRNA(GAG) was constructed to rescue the triple tRNA^{Leu}(GAG) knockout strain. The gene TL(GAG) was amplified by PCR as a 700-bp DNA fragment containing the natural promoter. It was subsequently cloned in pRS317 and the anticond GAG was mutated to UAG. The resulting plasmid (pRS317-C) was expressing a chimeric tRNA exhibiting the main body of tRNA^{Leu}(GAG) and a UAG anticodon. The plasmid could efficiently complement the triple tRNA^{Leu}(UAG) knockout strain. It was particularly useful during the northern blot analyses of tRNA^{Leu}(UAG) mutants since it did not hybridize with the probe designed to recognize tRNA^{Leu}(UAG).

In situ disruption of the genes encoding tRNA^{Leu}(GAG) and tRNA^{Leu}(UAG)

Disruption of the gene for tRNA^{Leu}(GAG) was performed as follow. First, a DNA fragment carrying the 1.5-kb DNA fragment of HIS3 gene ligated to the flanking sequences of tRNA^{Leu}(GAG) gene was constructed. For this, two PCR amplifications were performed by LA-Taq enzyme on yeast genomic DNA. A 1-kb DNA fragment located upstream of the tRNA gene was amplified by forward primer 5′-AAAGGATCTCATGTTGCTGATTGCTATCCC-3′ (BamHI in italics) and reverse primer 5′-AAACCGGCGCAATTCTATGTTGGGTA A-3′ (NotI in italics). A second 1-kb DNA fragment located downstream was amplified by forward primer 5′-AAATCTAGAGAATCTACGCATCTTC AA-3′ (XbaI in italics) and reverse primer 5′-AAAGGGCCCAATCTGCTGCTGTTGTA A-3′ (NotI in italics). The amplified upstream and downstream fragments were cleaved by BamHI + NotI and by XbaI + AphI, respectively. A 1.5-kb marker gene HIS3 was amplified by PCR from pRS313 by using forward primer 5′-AAAGGCGCCCGTCGCCGT TTCCGGTGATGATC-3′ (NotI in italic) and reverse primer 5′-AAATCTAGAGAATCTACGCATCTTC AA-3′ (XbaI in italic). The HIS3 gene was cleaved by NotI and XbaI. The three fragments above were ligated between the BamHI and AphI of pcDNA3 to generate a recombinant plasmid (PCDNSA-R) in which the recombinant DNA fragment insert [designated UHD or tl(gag):HIS3] consisted of the HIS3 marker gene in between the upstream and downstream DNA fragments of the yeast tRNA^{Leu}(GAG) gene (Supplementary Figure S1). The recombined fragment was excised by BamHI and AphI from pcDNA3-R, and the linear DNA fragment was introduced into the yeast diploid strain YPH501. Yeast transformants were grown on SD-His− plates at 30°C for 2–3 days. It was expected that the selected His+ colonies were exhibiting a tRNA^{Leu}(GAG) gene replaced by a HIS3 marker gene after homologous recombination with the UHD DNA fragment. To verify this hypothesis, sporulation of His+ colonies was induced on appropriate plates (1% potassium acetate, 0.1% yeast extract and 0.05% glucose). Tetrad were dissected and the resulting haploids were plated on YPDA plates (1% yeast extract, 2% peptone, 2% glucose, 0.004% adenine and appropriate agar) and further analyzed (described earlier). The yeast strain deleted for tRNA^{Leu}(GAG) gene was named tl(gag)-A1.

We used the same method to delete the three genes for tRNA^{Leu}(UAG), and the yeast strain was named tl(uag)-A1-3.

Construction of the random mutation library and screening by plasmid shuffling

Random mutagenesis of tRNA^{Leu}(UAG) was performed by hydroxylamine treatment. Plasmid pRS314-R (Trp′) was incubated in an appropriate volume of mutagenesis buffer (0.8 M hydroxyamine-HCl, 50 mM sodium phosphate, pH 6, 1 mM EDTA) at 37°C for 48 h to statistically obtain only one mutation per tRNA gene (29). Then, the DNA solution was extracted by phenol/chloroform, and precipitated with ethanol. The library was used to transformed tl(uag)-A1-3 strain in which the three genes for tRNA^{Leu}(UAG) were deleted. Transformants were grown at 30°C on plates of SD medium minus tryptophan (SD − Trp′) for 2–3 days. Then, transformants were replicated to SD − Trp′ plates containing 5-FOA and uracil to allow shuffling of the pALR-T maintenance plasmid [containing URA3 gene whose product converts 5-FOA to toxic 5-fluourouridine monophosphate (5-FUMP)]. The lethal clones were selected. The DNA was extracted for sequencing of the tRNA^{Leu}(UAG) mutated gene in pRS314-R.

Complementation assays by site-directed mutated tRNA^{Leu}

Plasmids carrying the site-directed mutated tRNA^{Leu}(UAG) genes were introduced separately into the tRNA knockout strain and transformants were grown at 30°C and treated as above with the randomly mutatated library. Transformants were subjected to drop-test on 5-FOA containing plates. The growth of the yeast cells was then observed and calculated (19,22).

Measurement of the growth rate

Yeast cells containing the different tRNA mutations were grown in liquid SD − Trp− medium. Each culture was diluted in liquid media to an initial A600 = 0.1. The diluted culture was shaken at 30°C. The A600 value was measured at various intervals, and the growth rate was calculated according to the logistic growth equation (30).

Analysis of in vivo aminoacylation by northern blot

The yeast cell knockout strains containing different mutations were grown in liquid SD − Trp− media to A600≈0.6, and then were harvested by centrifugation. Cell pellets were suspended in 1 ml of extraction buffer (0.3 M sodium acetate, pH 4.5, 10 mM EDTA) and
extracted with one volume of phenol extraction (pH 4.5). The supernatant was then re-extracted with one volume of phenol/chloroform (pH 4.5). Three volumes of 100% ethanol were mixed with the supernatant to precipitate the RNA, and the final precipitate was dissolved in 50 μl of storage buffer (10 mM sodium acetate pH 4.5).

tRNAs were separated by acid 12% PAGE containing 8 M urea. Thirty micrograms of tRNA samples were loaded on a 0.4-mm thick gel in 0.1 M sodium acetate buffer pH 5. Electrophoresis was carried out at 4°C and constant 12 W (∼400 V) for 24 h in 0.1 M pH 4.5 sodium acetate buffer. The portion of the gel containing the tRNAs was transferred to a nylon membrane (Millipore, Bedford, MA, USA) by electrophoresis at constant 100 V for 30 min in 0.5× TBE buffer. The nylon membrane was then baked for 30 min at 80°C (19,31).

Pre-hybridization was performed at 65°C for 6 h in 15 ml of solution (10× Denhardt’s solution, 6× SSC and 0.5% SDS). Hybridization was performed at 65°C for 6 h in 15 ml of the same solution, in the presence of a tRNA\textsubscript{Leu\textsubscript{e}}(UAG)\textsuperscript{Lys} probe (5’-AGGA CTTCA ACCCT TGCAT CCGA AGGCT C-3’) as an internal control) labeled with digoxigenin at each 5’-end. Membranes were washed at 65°C in a 2% SSPE, 0.5% SDS solution for 20 min, hybridized with anti-digoxigenin-AP and visualized with CDP-star, following standard procedures (32). Signals were quantified using a Fuji Bioimager Bas2000.

Preparation of proteins and tRNA\textsubscript{Leu}\textsubscript{e}S

The ycLeuRS and its mutant ycLeuRS-D419A were purified as described (22). Escherichia coli strain BL21(DE3) was transformed with plasmids pET28-yceLeu\textsubscript{S} and pET28-yceLeu\textsubscript{S}-D419A which were constructed in our laboratory (22). Transformants were grown in 500 ml Luria–Bertani liquid medium (1% peptone, 0.5% potassium phosphate buffer (pH 7.5) containing 50% glycerol, 250 mM imidazole) and 2.5 mg of yeast total RNA were mixed and incubated at room temperature for 1 h. The mixture was washed with 1 ml of NTE buffer (5 mM Tris–HCl pH 8.0, 2.5 mM EDTA and 200 mM NaCl) at room temperature, and tRNA was eluted with 1 ml 0.1× NTE buffer at 65°C. The tRNA was further purified by electrophoresis on a 10% polyacrylamide/8 M urea gel, followed by passive elution in 10 mM Tris–HCl (pH 7.5) containing 0.3 M NaCl and 0.5 mM EDTA, and precipitation by ethanol after phenol–chloroform extraction. Eluted tRNA\textsubscript{Leu\textsubscript{e}}(UAG)\textsubscript{S} were renatured by heating at 80°C for 5 min and slow cooling to room temperature (10).

Aminoacylation, decacylation and AMP formation

The aminoacylation assay of WT yctRNA\textsubscript{Leu\textsubscript{e}}(UAG) and its mutants were determined at 30°C in a reaction mixture containing 60 mM Tris–HCl (pH 7.5), 10 mM MgCl\textsubscript{2}, 2 mM dithiothreitol, 4 mM ATP, 20 μM [\textsuperscript{3}H]leucine, 2 μM tRNA\textsubscript{Leu\textsubscript{e}} and 1 nM ycLeuRS. Kinetics constants for WT yctRNA\textsubscript{Leu\textsubscript{e}}(UAG) and its mutants were determined in the reaction mixture mentioned above, in the presence of various concentrations (from 0.05 to 2 μM) of the relevant yctRNA\textsubscript{Leu\textsubscript{e}}(UAG) substrates.

To determine the effect of EF-1α on leucine charging activity, aminoacylation was performed in the presence of a mix of 0.1 mg/ml EF-1α and 4 mM GTP (pre-incubated with pyruvate kinase for 30 min). In parallel, a negative control was performed in which an equal concentration of BSA and GTP was added.

Aminoacylated [\textsuperscript{3}H]Leu-tRNA\textsubscript{Leu\textsubscript{e}}(UAG) WT and mutants were obtained by charging the corresponding tRNAs with Leu in the presence of WT ycLeu\textsubscript{S}. The spontaneous hydrolysis levels of Leu-tRNA\textsubscript{Leu\textsubscript{e}}(UAG)\textsubscript{S} were measured in 60 mM Tris–HCl (pH 7.5), 10 mM MgCl\textsubscript{2}, 1 μM [\textsuperscript{3}H]Leu-tRNA\textsubscript{Leu\textsubscript{e}}(UAG)\textsubscript{S}, with 0.1 mg/ml EF-1α and 4 mM GTP or with equal concentration of BSA and GTP at 30°C. Aliquots (10 μl) of reaction solution were quenched on Whatman filter pads at different times. Radioactivity was quantified in a scintillation counter (Beckman, Pasadena, CA, USA).

Misacylated Ile-tRNA\textsubscript{Leu\textsubscript{e}}(UAG) WT and mutants were obtained by charging with Ile in the presence of ycLeu\textsubscript{S}-D419A (22). The editing activities of ycLeu\textsubscript{S} toward mischarged [\textsuperscript{3}H]Ile-tRNA\textsubscript{Leu\textsubscript{e}} and mutants were determined in the reaction mixture mentioned above, in the presence of various concentrations (from 0.05 to 2 μM) of the relevant mischarged yctRNA\textsubscript{Leu\textsubscript{e}}(UAG) substrates.
measured at 30°C in 60 mM Tris–HCl (pH 7.5), 10 mM MgCl₂, 2 mM dithiothreitol and 1 μM [³H]Ile-tRNA⁰⁰⁰ and mutants. The reactions were initiated with 1 nM ycLeuRS.

Because the editing reaction consumes ATP, it can be measured using the breakdown of ATP (to AMP and PPi) in the presence of non-cognate amino acids (4,6). AMP formation was measured in reaction mixtures containing 60 mM Tris–HCl (pH 7.5), 10 mM MgCl₂, 5 mM DTT, 3 mM ATP, 20 nM [α-³²P]ATP, 15 mM Nva, 5 U/ml pyrophosphatase, in the presence or absence of 5 μM tRNA. The reactions were incubated at 30°C and initiated by 1 μM ycLeuRS. Aliquots (1.5 μl) were quenched in 6 μl of 200 mM sodium acetate (pH 5.0). Quenched aliquots (1.5 μl) were dripped into a TLC plate. Nva-[³²P]AMP, [³²P]AMP and [³²P]ATP were separated in 0.1 M ammonium acetate and 5% acetic acid. The plates were visualized by phosphor imaging and the data were further analyzed (5).

RESULTS
Deletion of the gene for tRNA⁰⁰⁰(GAG) does not affect cell viability

The genome of S. cerevisiae contains 21 tRNA⁰⁰⁰ genes distributed as 10 tRNA⁰⁰⁰(CAA) genes, seven tRNA⁰⁰⁰(UAA) genes, three tRNA⁰⁰⁰(UAG) genes and one tRNA⁰⁰⁰(GAG) gene [TL(GAG)]. Based on the assumption that the single-copy TL(GAG) for tRNA⁰⁰⁰(GAG) should be essential according to the decoding capacity of the anticodon GAG (35), we first deleted the TL(GAG) gene in the diploid YPH501 strain. The TL(GAG) gene is located between positions 700 679 and 700 760 in chromosome VII and is flanked by a tRNA⁰⁰⁰(UUU) gene and two genes YGR106C and YGRWDELTA19 (Figure 1A) (35). By the method described above, plasmid pcDNA3-R was constructed carrying HIS3 flanked by the upstream and downstream sequences of TL(GAG) (so called UHD fragment). The linear BamHI-Apal UHD fragment was introduced into the yeast strain YPH501. In this way, the 0.1-kb TL(GAG) gene was substituted by the 1.5-kb HIS3 gene, keeping the recombination flanking sequences intact. Transformants were grown on SD/His- plates in order to select recombination events. To confirm that the diploid yeast transformants had lost TL(GAG) as expected, we examined the corresponding region of the chromosome by PCR amplification and sequencing (Invitrogen, Shanghai, China). Two DNA fragments were PCR-amplified from primers hybridizing to the flanking sequences on one side and to HIS3 on the other side (Figure 1A). PCR products of 2.1-kb and 1.6-kb sizes were amplified as expected from the strains carrying the UHD gene modification. Additionally, the presence of NotI and XbaI sites resulting from the insertion of HIS3 was verified by digestion, producing 1.05-kb and 1+0.6-kb fragments, respectively (Figure 1B). These

Figure 1. Strategy of construction and characterization of the tRNA⁰⁰⁰(GAG) knockout strain. (A) Schematic map of the chromosomal TL(GAG) region and of the UHD DNA fragment constructed to exchange the TL(GAG) gene. Arrows symbolize the PCR primers used to verify the knockout experiment. Predicated sizes and restriction sites are indicated. (B) Agarose gel of the PCR products of YPH501 recipient strain and tl(gag)::HIS3 strain. Lane 1: 100 bp plus DNA ladder (Transgene, Beijing, China); lanes 2 and 3: PCR products amplified with tl(gag)::HIS3 genomic DNA. Lanes 4 and 5: PCR products amplified from the recipient YPH501 yeast strain. Lane 6: PCR product from lane 2 digested with NotI. Lane 7: PCR product from lane 3 digested with XbaI. (C) Growth of strains on YPDA plate. Parental strain YPH501 [a/a TL(GAG) +/+], the knockout diploid strain [a/a TL(GAG)/tl(gag)::HIS3] and the haploid knockout strain [tl(gag)::HIS3] are equally growing on YPDA. (D) Northern blot analysis on the RNA content of the yeast strains. 5S RNA was detected as an internal control. The tRNA probe was the following 5'-CCC GC GCCUC AGAAG AGAUC AGGAC CUCGA-3'.
data confirmed that the UHD fragment was inserted into the yeast strain. However, after dissecting the tetrads obtained after incubation of the diploid strain on sporulation medium, two of the spores were His\(^+\) as WT haploids, while the other two haploids which lacked TL(GAG) were exhibiting a His\(^-\) phenotype. We found that the haploids knockout strain could grow perfectly on YPDA plates (Figure 1C). These data suggested that tRNA\(^{Leu}(GAG)\) may not be an essential tRNA in yeast and that its decoding capacity overlaps with another tRNA\(^{Leu}\). To verify that tRNA\(^{Leu}(GAG)\) was not produced any more in the knockout strain, we performed a northern blot analysis on the haploid His\(^-\) strain and indeed no such tRNA could be detected (Figure 1D). In addition, we compared the growth rate of the knockout strain [named tl(uag)-1] and WT YPH499 strain in different conditions. At 23\(^\circ\)C, 30\(^\circ\)C and 37\(^\circ\)C, on YPDA medium, similar growth rates were observed (Supplementary Figure S2). When grown on YPDA with excess NaCl or glycerol, no difference was observed between the strains (data not shown) (36). Altogether, the data showed that the gene TL(GAG) of tRNA\(^{Leu}(GAG)\) may be a dispensable gene and thus it is not a suitable gene to construct a strain dedicated to genetic selection of tRNA mutants.

**Construction of triple knockout strain of the tRNA\(^{Leu}(UAG)\) genes**

The triple deletion of tRNA\(^{Leu}(UAG)\) genes was performed in the haploid strain YPH499 [which was chosen due to the simple genetic manipulations with having fewer spores to dissect (37)]. tRNA\(^{Leu}(UAG)\) is transcribed from three TL(UAG) copies, two of them located on chromosome XII and one on chromosome X. Considering that TL(UAG) might be essential, a rescuing plasmid pALR-T [tlR4, URA, ADE3, TL(UAG)] was first introduced into YPH499. The deletion of the three copies of TL(UAG) was carried out successively as described above, adding sequentially the HIS3, LEU2 and ADE2 genes. PCR- and sequencing analysis confirmed that the genes were deleted and replaced by the auxotrophic markers (Figure 2A–F). The deleted strains were named tl(uag)-\(1,2\) and tl(uag)-\(1,2,3\). A plasmid-shuffling assay was performed on plates containing 5-FOA to check if the rescuing plasmid pALR-T could be shuffled from these strains. On 5-FOA plates, strains tl(uag)-\(1,2\) and tl(uag)-\(1,2,3\) were perfectly growing as the WT strain indicating that the rescuing plasmid was not essential. After deletion of the third tRNA\(^{Leu}\) gene [tl(uag)-\(1,2,3\)], the strain was unable to grow on 5-FOA-containing medium, indicating that the rescuing plasmid could not be lost (and thus 5-FOA was metabolized in 5-FUMP toxic for cells) (Figure 2G). This strongly suggests that the triple knockout experiment was successful and that tRNA\(^{Leu}(UAG)\) is an essential tRNA\(^{Leu}\) which has a decoding capacity that cannot be covered by another tRNA\(^{Leu}\). Northern blot analysis further confirmed that the tl(uag)-\(1,2,3\) strain was not containing any tRNA\(^{Leu}(UAG)\) and that the triple knockout was successful (Figure 2H). Altogether, the data showed that the knockout of the three tRNA\(^{Leu}(UAG)\) genes is lethal for the yeast cells. The triple mutant can be complemented by a plasmid copy of the rescuing tRNA and a shuffle assay can be set up to select and test mutated tRNA\(^{Leu}(UAG)\).

**Site-directed mutagenesis to test the triple tRNA knockout strain**

We mutated several well-defined tRNA\(^{Leu}\) identity elements to validate the use of strain tl(uag)-\(1,2,3\) for *in vivo* studies. The discriminator base A73, a positive identity determinant for recognition by LeuRS, and the crucial G18-Ψ55 base pair for maintaining the tertiary structure of tRNA\(^{Leu}\) were chosen (9,10). These two elements of tRNA\(^{Leu}\) are necessary for aminoacylation in most species. Three mutations, A73C, G18U and Ψ55G were constructed and the mutated genes was introduced into strain tl(uag)-\(1,2,3\). Then, the three transformants were plated on 5-FOA-containing medium and no one could grow, confirming that the mutations induced lethality of the cells. Another mutant, Ψ55C, which allowed formation of the tertiary base pair with G18 could grow normally at the presence of the 5-FOA (Figure 3 and Supplementary Figure S3). These results matched those of former *in vitro* data and strongly supported that strain tl(uag)-\(1,2,3\) as an efficient system for *in vivo* investigations on tRNA\(^{Leu}\) identity determinants.

**Essential nucleotides revealed by screening of a randomly mutated library**

To identify the essential nucleotides of tRNA\(^{Leu}\), a randomly mutated library of plasmid pRS314-R [Trp2, TL(UAG)] was generated by chemical modification by hydroxylamine that preferentially induces G→U or C→A transversions (29). The library was introduced into strain tl(uag)-\(1,2,3\) rescued by pALR-T and the transformants were grown in SD—TRP plates. Five thousand colonies were obtained. They were replicated on 5-FOA-containing medium in order to induce pALR-T shuffle. A total of 11 clones did not grow on 5-FOA-containing medium, meaning that the shuffle could not occur because pRS314-R contained a TL(UAG) gene unable to sustain cell growth or grew slowly. Plasmids from the 11 clones were extracted. The phenotypes were confirmed by a second transformation and plating on 5-FOA, then the plasmid DNA was sequenced.

Among the 11 mutations, 9 appeared within TL(UAG) whereas the 2 others were located in another part of the plasmid outside of the tRNA gene. Among the lethal mutations were G18U in the D-loop, G36U and G37U (two occurrences) in the anticodon loop, C61A and C62A (two occurrences) in the T-stem. Among the mutants that grew slowly, one was harboring a G2U mutation in the acceptor stem (70% of WT of the growth rate) and the second one a mutation G30U in the anticodon stem (inducing a 45% growth decrease) (Figure 3A). We grew yeast cells containing the mutants of tRNA\(^{Leu}\) on both solid and liquid media to further confirm the phenotype. On solid medium, lethal or slow growth phenotype was observed directly (Supplementary Figure S3). In liquid medium, the \(A_{600}\) value of the culture...
Figure 2. Construction of the tRNA<sup>Leu</sup>(UAG) triple knockout yeast strain. (A, C and E) Schematic map of the chromosomal regions containing the three tRNA<sup>Leu</sup>(UAG) genes and of the homologous recombination DNA fragment constructed to delete the TL(UAG) genes. Arrows symbolize the PCR primers used to verify the knockout experiment. Predicated sizes and restriction sites are indicated. (B, D and F) Agarose gels of the PCR products of YPH499 strain and tRNA<sup>Leu</sup> deleted strains. Lanes 1: DNA ladder; lanes 2 and 3: PCR products amplified with genomic DNAs from deleted strains. Lanes 4 and 5: PCR products from lanes 2 digested with NotI or BamHI. Lanes 6: PCR products from lanes 3 digested with XbaI, XhoI or EcoRI. (G) Parental strain (YPH499), derivative strain WT-R (transformed pALR-T into YPH499), single knockout strain tl(uag)<sup>1-3</sup>::HIS3/LEU2 and triple knockout strain tl(uag)<sup>1-3</sup>::HIS3/LEU2/ADE2 were grown on a normal YPDA medium and then spread over on a plate containing 5-FOA. (H) Northern blot analysis on the RNA content of the three knockout strains. 5S RNA was detected as an internal control. The tRNA probe was the following 5'-CCUUG CAUCC GAAGA UAUCA GAGCC UAAAU-3'.

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was measured at various intervals, and the growth rate was calculated (Figure 3B).

Surprisingly, tRNALeu(UAG) harbor a long variable arm carrying six G or C nucleotides that could have been mutated by hydroxylamine, however, no lethal mutation was selected in the variable arm. It is known that this tRNA subdomain is a crucial element for proper tRNA-enzyme recognition, which involves more structural constraints than sequence-specific interactions with the synthetase (38). Therefore, single–site mutations may not disrupt or affect the LeuRS:tRNALeu complex network of constraints.

Effects of the mutations on the steady state and aminoacylation levels of tRNALeu

Mutations in the tRNA primary sequences may impact several processes, such as transcription, stability, bases modification, aminoacylation, editing and interaction with the translation factors and ribosome (39). Some tRNAs are also involved in signal transduction as cellular factors (40). To identify the mutations that affect the steady state and aminoacylation status of tRNALeu, we performed northern blots analyses. By extracting the tRNAs in acidic conditions, we could preserve the aminoacyl-ester bond and analyze the levels of in vivo aminoacylation (31).

To rescue the cells harboring a lethal tRNALeu mutants, we complemented the cells with a chimeric tRNALeu consisting of the body of tRNALeu(GAG) in which the codon was changed to (UAG). This tRNA was expressed from the rescuing plasmid pRS317-C. In that way, we could rescue the cells and analyze the mutated tRNALeu(UAG) content by northern blot without hybridizing the rescuing plasmid. Total tRNAs extracts were prepared and the tRNALeu(UAG) levels were analyzed (19).

Almost all of the 11 mutations from site-directed and random mutagenesis affected the aminoacylation levels of tRNALeu(UAG). Mutant A73C was totally uncharged in vivo, which is consistent with in vitro results (10,34). In vitro, the long-distance interaction between G18 and Ψ55 is important to maintain the tertiary structure and the aminoacylation activity. The present work shows that preventing the interaction with G18U or Ψ55G mutations led to total uncharging of the tRNA by LeuRS (Figure 4). Mutation Ψ55C, which theoretically allowed formation of hydrogen bonds between bases 18 and 55, restored partial aminoacylation of tRNA (Figure 4), suggesting that the structure, the specific nucleotides or their modifications play a role during the aminoacylation of tRNA.

The two mutations of G2U and G30U of yeast tRNALeu(UAG) exhibiting a reduced growth rate in the triple tRNA knockout strain were only partially aminoacylated in vivo (Figure 4). The G2U mutation significantly reduced the aminoacylation level (~40% compared of WT), suggesting that the modification of the geometry of the acceptor arm by changing the original G:U wobble base pair is responsible of the aminoacylation defect. In addition, we cannot exclude that G2 is a determinant of the leucine identity. On the other hand, G30U was ~70% as aminoacylated as the WT tRNALeu, suggesting that the mutation more likely impacted the translation process (Figure 4).

The lethal mutations C61A and C62A located in the T-loop led to drastically reduced tRNALeu(UAG) levels (Figure 4), suggesting that the transcription process was deficient or the stability of the transcripts reduced. Indeed, tRNAs are RNA polymerase III transcripts and the ICR (internal control region) box is located into the T-loop (41), thus we cannot exclude that mutations C61A and C62A disturb the transcription process.

Although the G36U mutation changed the anticodon of tRNALeu(UAG) from leucine anticodon UAG to serine anticodon UAU (thus switching the decoding property of the tRNA), the mutant of tRNALeu could still be charged with an amino acid by LeuRS to a significant level (34) (Figure 4). However, the mutation was lethal as expected due to the swap of decoding properties from leucine to serine codons. Surprisingly, mutation G37U totally abolished aminoacylation, indicating that G37 residue is essential for the aminoacylation of tRNALeu (34) (Figure 4).
It has been reported that A35 of yeast tRNA\textsubscript{Leu}(UAG) is crucial for aminoacylation of tRNA. Introducing an A35G was found to reduced the $k_{\text{cat}}$ and $K_m$ of tRNA\textsubscript{Leu}(UAG) dramatically \textit{in vitro} (34). In the present investigation we did not select any mutant of A35, probably because adenine residues are poorly reactive to hydroxylamine. Thus, we constructed the A35G mutation and introduced it into the triple tRNA-knockout strain. The mutation induced a lethal phenotype on 5-FOA plate (Figure 3 and Supplementary Figure S3) as expected since the decoding property was shifted from Leu to Pro. We analyzed the \textit{in vivo} aminoacylation level of the mutant and found that the aminoacylation level was decreased by about 30% (Figure 4), which is an effect significantly different from the \textit{in vitro} reported data (total abolished aminoacylation) (34). Thus, the anticodon mutations affected aminoacylation slightly; indicating that A35 or G36 are not critical elements of the aminoacylation reaction, which is the universal recognition model between LeuRS and tRNA\textsubscript{Leu} from various species in many \textit{in vitro} studies (9,10).

The anticodon stem is involved in editing function of LeuRS

LeuRS contains an editing domain that deacylates misacyl-tRNA that could alter the fidelity of the translation process. Under usual growth conditions, yeast cells are not exposed to an excess of non-cognate amino acids and the editing function is hardly observable. However, when yeast cells are exposed to an excess of non-cognate amino acids, mis-aminoacylation of tRNA occurs and editing becomes essential for the viability (5,22). To assay the editing properties, we replicated the previous 5000 transformants of the triple knockout strain tl(uag)-D\textsubscript{1-3} on plates containing 10 mM norvaline (Nva), an analog of leucine (22). We identified a single clone showing a reduced growth rate on 5-FOA plates. The tRNA\textsubscript{Leu} gene was exhibiting a G42U mutation (Figure 5A and Supplementary Figure S4). A dose-dependent assay was set up in the present of various concentrations of Nva to further confirm that G42U mutant was sensitive to 5-FOA (Figure 5A and Supplementary Figure S4). In the presence of 3 mM Nva, the yeast strain harboring the G42U mutation grew slower than the WT in the same conditions (Supplementary Figure S4). When northern blots were performed on tRNA extracts prepared in acidic conditions, a complete aminoacylation of G42U mutant was observed (Figure 5B). However, Leu-tRNA\textsubscript{Leu} and
Nva-tRNA$^{\text{Leu}}$ are very similar molecules that cannot be distinguished by PAGE. Thus, we concluded that G42U mutation does not prevent the aminoacylation by Nva or Leu but more likely affect the editing of Nva-tRNA$^{\text{Leu}}$, therefore leading to growth rate defects on 5-FOA plates.

Because G42 forms a strong G:C base pair with C28, it might strengthen the stability of the anticodon stem which may be critical for the editing activity. To test this hypothesis, we further constructed a double mutant C28A/G42U exhibiting a weaker A:U base pair. The in vivo aminoacylation level of the double mutant was not affected and was similar to the single G42U mutant (Figure 5B) while the growth effect recovered to some extent as compared with the G42U single point mutation tRNA$_{\text{Leu}}^{\text{UAG}}$(UAG) (Figure 5A and Supplementary Figure S4).

Altogether, the results suggest that the anticodon stem plays a role during the editing reaction. A perfect base pairing for a correct geometry and/or a high thermodynamic affinity seem to be needed in the anticodon stem of tRNA$_{\text{Leu}}^{\text{UAG}}$ for an optimal editing activity catalyzed by LeuRS.

**Aminoacylation, deacylation and EF1-α protection assays on tRNA$_{\text{Leu}}^{\text{UAG}}$(UAG)s**

Yeast tRNA$_{\text{Leu}}^{\text{UAG}}$(UAG) WT and three representative mutants (A35G, G37U, G42U) were purified from yeast transformants containing their genes using biotin-Streptavidin Agarose Resin. The partially purified tRNA$_{\text{Leu}}^{\text{UAG}}$(UAG)s were further separated on a denaturing gel. The final tRNAs looked homogeneous in the gel (Supplementary Figure S5). The plateau value of WT and its two mutants A35U and G42U reached 1600 pmol/A260, however, the mutant G37U could not be aminoacylated (Supplementary Table S1). This is consistent with the undetectable in vivo growth rate (Figures 3, 4 and 5B). Kinetic constants of ycLeuRS for WT, A35U and G42U mutants of tRNA$_{\text{Leu}}^{\text{UAG}}$(UAG) were assayed, which showed that the catalytic efficiency of ycLeuRS for these tRNA$_{\text{Leu}}^{\text{UAG}}$s was not obviously changed. However, leucylation of G37U mutant was abolished completely (Table 1) which corroborates the in vivo results (Figures 3, 4A and 5B). Because modified G37 in many tRNA$_{\text{Leu}}^{\text{UAG}}$s is crucial to their leucylation (42), G37U mutation must change the modification of G37, and our results further indicated the importance of modified G37 in tRNA$_{\text{Leu}}^{\text{UAG}}$.

In vivo, the ester linkage between the amino acid and tRNA is labile and is readily hydrolyzed if the aa-tRNA is not recognized and bound by elongation factor. To eliminate the possibility that some mutated tRNAs have lost their binding capacity to elongation factor in vivo, we cloned the gene encoding yeast EF-1α (33), purified the protein and carry out charging and protection assays. In the presence or absence of EF-1α, leucylation of tRNA$_{\text{Leu}}^{\text{UAG}}$(UAG) WT and its three mutants A35G, G37U and G42U were assayed. The data showed that their accepting activities were not improved by the presence of EF-1α (Figure 6A). Moreover, we assayed the protection of aminoacylated Leu-tRNA$_{\text{Leu}}^{\text{UAG}}$ by EF-1α from hydrolysis in water in the absence of ycLeuRS. Under physiological conditions (pH 7.5), the ester linkage between Leu and tRNA$_{\text{Leu}}^{\text{UAG}}$(UAG) was moderately hydrolyzed and the presence of EF-1α only slightly improved the stability of the ester linkage (Figure 6B). No obvious difference was observed between WT tRNA$_{\text{Leu}}^{\text{UAG}}$(UAG), A35G and G42U mutants indicating that the lethal phenotype of the mutants is not due to a loss of protection by EF-1α in vivo.

Our in vivo experiment reveals that excess Nva decreases the growth rate of yeast cells harboring the
tRNA\textsuperscript{Leu}(UAG)-G42U mutant. Our previous work also elucidated that the Nva could be mischarged and incorrect proteins were generated (22). An \textit{in vitro} editing assay was performed to investigate whether Nva could be charged to tRNA\textsuperscript{Leu}(UAG)-G42U. Although no Nva-tRNA\textsuperscript{Leu}(UAG)-G42U was accumulated when catalyzed by ycLeuRS (data not shown); the hydrolytic activity of ycLeuRS to mischarge Ile-tRNA\textsuperscript{Leu}(UAG)-G42U was reduced (Figure 7A), and in the presence of tRNA\textsuperscript{Leu}(UAG)-G42U and Nva, AMP formation was decreased (Table 2, Figure 7B and C) Additionally, Nva-AMP formation was increased (Figure 7B and C) as compared to tRNA\textsuperscript{Leu}(UAG) WT. Further, tRNA\textsuperscript{Leu}(UAG)-A35G revealed a slight decrease in editing activity. The \textit{in vivo} and \textit{in vitro} data both indicated that A35 and G42 at the anticodon stem and loop are important for editing.

DISCUSSION

\textit{Saccharomyces cerevisiae} is using superwobbling to read leucine codons

The anticodon of a tRNA molecule pairs by hydrogen bonding with a codon in mRNA. Pairing between the second and third positions of anticodons with the second and first positions of codons follows the usual rules of base pairing proposed by Crick (43), with some play or ‘wobble’ between the first anticodon base and the third codon base during pairing in order to expand the coding capacity in protein synthesis. For example, the base pair between base 34 of tRNA and the third base of triplet codon of mRNA may be Watson–Crick pairing G34–C3, U34–A3 and wobble base pairing G34–U3, U34–G3. Since the original hypothesis of Crick, more and more wobble base pairings between anticodon and codon have been identified and they differ in various organisms and systems. Several modifications of the first anticodon base also participate in pairing (44)—For instance, in \textit{E. coli}, tRNA\textsuperscript{Leu}(UAG) translates CUA, CUU, CUG, but not CUC \textit{in vitro} due to a uridine-5-oxyacetic acid modification (45). Modification of bases may also have opposite effects and expand the decoding capacity. tRNA\textsuperscript{Val}(UAC) of \textit{E. coli} and tRNA\textsuperscript{Pro} (UGG) of \textit{Salmonella}, contain a 5-oxyacetic acid modified U34 which helps to decode a degenerate codon box (46). The structural basis for the expansion of the decoding capacity of tRNA by modification of uridines was recently solved (47). In addition it is now evident that unmodified U34 pairs with all four bases in third position of codons (in UNN family boxes). Thus, a single anticodon with a general formula UNN is potentially able to translate the four codons in its family box. The phenomenon, called ‘superwobbling’ represents one possible mechanism of reduction of in number of tRNAs (48). This form of translation takes place in \textit{Mycoplasma} and some mitochondria and chloroplasts (49). However, if superwobbling facilitates translation with a reduced set

![Figure 7. In vitro editing the mischarged tRNA\textsuperscript{Leu}(UAG) WT and mutants by ycLeuRS and the AMP and Nva-AMP formations with the corresponding tRNAs. (A) Deacylation assays catalyzed by ycLeuRS (1 nM) of 1 μM of Ile-tRNA\textsuperscript{Leu} WT (filled circle), -A35G (filled triangle), -G42U (filled square), reaction without enzyme (filled diamond) was performed as spontaneous hydrolysis control. (B) TLC plates showing the [32P]AMP and Nva-[32P]AMP formation catalyzed by ycLeuRS (1 μM). (C) Quantification of the AMP or Nva-AMP formation with tRNA\textsuperscript{Leu}(UAG) -WT (filled circle), -A35G (filled triangle), -G42U (filled square) and without tRNA (filled diamond), respectively. 's values of AMP and Nva-AMP formations are calculated from the slopes and reported in Table 2.

Table 2. Observed steady-state constants of ycLeuRS in AMP and Nva-AMP synthesis reaction in the presence of Nva and ycRNA\textsuperscript{Leu}(UAG) WT or its mutants

| Mutants of tRNA | AMP formation | Nva-AMP formation |
|-----------------|---------------|-------------------|
|                 | k\textsubscript{obs} (s\textsuperscript{-1}) | (relative) k\textsubscript{obs} (s\textsuperscript{-1}) | (relative) |
| WT              | 1.93 ± 0.23   | 1.00 (7.20 ± 0.56) | 1.00    |
| A35G            | 1.41 ± 0.08   | 0.73 (1.25 ± 0.13) | 1.74    |
| G42U            | 0.74 ± 0.10   | 0.38 (2.32 ± 0.65) | 3.22    |
| No tRNA         | 0.30 ± 0.03   | 0.16 (5.16 ± 1.71) | 7.17    |

All rates represent the average of three trials with the standard deviations indicated.
of tRNAs, it has to pay the price of a reduced translation efficiency compared to most organisms that prefer more than one anticodon per family box (48).

The data here presented showed that the gene for tRNA_{Leu}^{Leu}(GAG) could be deleted without impairing yeast growth, whereas the knockout of the gene of tRNA_{Leu}^{UAG} was lethal. This suggests that CU(C/U) codons were decoded by another tRNA which should obviously be tRNA_{Leu}^{UAG}. Thus, the ability of yeast tRNA_{Leu}^{UAG} to translate the four codons might imply a superwobble mechanism as observed with tRNAs carrying unmodified U34 (50). Indeed, the spectrophotometric investigations performed on yeast tRNA_{Leu}^{UAG} showed that U34 was not modified and was even able to translate the six Leu codons in vitro (50). Thus, our finding that protein synthesis occurs in the absence of tRNA_{Leu}^{(GAG)} demonstrates that a single tRNA species with an unmodified U in the wobble position can accurately decode triplets with all four nucleotides in the third codon position, therefore supporting the superwobble hypothesis to sustain protein biosynthesis. Based on protein synthesis defects, it was proposed that superwobbling is less efficient than standard and wobble base-pairing, presumably because codon–anticodon recognition is delayed (48). Here, we did not observe any growth defect of the tRNA_{Leu}^{Leu}(GAG) knockout cells, however, we did not thoroughly analyze growth rates in different conditions, thus, we cannot exclude that superwobbling is less efficient than standard and wobble base-pairing.

An intriguing question is whether the capacity for superwobbling of tRNA_{Leu}^{UAG} carrying an unmodified U is used spontaneously in WT yeast cells or only in tRNA_{Leu}^{GAG} deleted cells. The capacity of reading may be latent in the WT cells and revealed by the tRNA deletion.

The six Leu codons are spread in a four-codon box (CUN) and a two-codon box (UUR). In S. cerevisiae, the usage of codons CUU, CUC, CUG, CUA, UUG and UUA is 12.9%, 5.8%, 11.2%, 14.2%, 27.8% and 28.1%, respectively (51). At the tRNA gene level, we can see that four tRNA genes are in charge of decoding the four-codon box and 17 tRNA genes for the two-codon box. Of interest, we can notice that CU(C/U) codons that are theoretically decoded by tRNA_{Leu}^{Leu}(GAG) represent 18.7% (12.9+5.8%) of the total number of Leu codons, which is a high value for a tRNA transcript from a single gene copy. For instance, tRNA_{Leu}^{Leu}(UAG) decoding CU(G/A) is transcribed from three genes and represents 24.4% (11.2+14.2%) of Leu codons. The extreme example is tRNA_{Leu}^{Leu}(CAA) which decodes UUG codons (27.8% of total Leu codons) from 10 copies of gene. Altogether, the data indicate that the ratio of codons to read per tRNA isoacceptor gene is the highest for tRNA_{Leu}^{(GAG)}, suggesting that superwobbling by tRNA_{Leu}^{Leu}(UAG) might occur in vivo as soon as a deficit of charged tRNA_{Leu}^{(GAG)} appears. However, it remains unclear whether superwobbling happens in normal growth conditions or only extreme conditions.

tRNA_{Leu}^{Leu}(UAG) triple knockout strain as a tool to select tRNA_{Leu}^{Leu} mutants in vivo

After construction of the triple tRNA_{Leu}^{Leu}(UAG) knockout strain, three loss-of-function mutations (A73U, G18U and Ψ55G) were constructed to test whether the strain could be used to isolate lethal mutations. The three mutations were chosen because they modified the main identity element (A73) or the tertiary structure of the tRNA (G18, Ψ55) that has been shown to be essential for Leu identity in almost all tRNA_{Leu} isoacceptors tested. In our assay, the triple knockout strain transformed with the mutated tRNA genes was unable to grow or survive because the cells could not shuffle the rescuing plasmid carrying the WT tRNA_{Leu} gene. The result of this first assay was lethality in agreement with the in vitro data that showed critical decreases of the aminoacylation efficiency of the three tRNA mutants (9,10). The result confirmed that the triple knockout strain and the rescuing plasmid could be used to detect tRNA mutations critical for aminoacylation or other functions of the tRNA.

We constructed a library of tRNA_{Leu} genes randomly mutated by hydroxylamine and used it to isolate tRNA mutations that induced lethality of the yeast knockout strain. Five thousand transformants were plated on minimal medium supplemented by the required amino acids and then replicated on plates containing 5-FOA. Clones those were unable to grow on the later medium contained lethal tRNA_{Leu} genes. Similarly, the 5000 clones were replicated on medium containing an excess of norvaline (Nva) in order to isolate tRNA_{Leu} mutants inducing a toxic effect in the presence of Nva. Nva is an analog of Leu that is misactivated and mischarged on tRNA_{Leu} by LeuRS and then proofread by the editing activity of the same enzyme (5,22). From this selection, it was expected to isolate tRNA_{Leu} mutants that impact the tRNA-dependent editing activity of LeuRS, leading to the incorporation of higher amounts of Nva into the proteins and then to toxicity.

As a result from the selections, we isolated tRNA_{Leu} mutants that induced effects that could be classified in three distinct groups as follows.

(i) The first group contained the two lethal mutations located in the T-arm: C61A and C62A. Northern blot analysis showed that the two tRNAs were perfectly aminoacylated, however the steady-state levels of the two tRNAs were severely decreased, to ~30% and 25% of the WT level for C61A and C62A, respectively. These low levels might result from a transcription defect since transcription by RNA polymerase III (41) is using an internal promoter called internal control region (ICR) located in both A box (nt 8–20) and B box (nt 51–63) (52). Thus, lethal mutations in positions 61 and 62 might affect transcription as already proposed for equivalent mutations isolated in tRNAArg and tRNASer in yeast (19,53). Alternatively, the low levels of tRNAs could result from a rapid degradation due to unstable tRNA structures. Indeed, the two mutations C61A and C62A introduced mismatches in the T-arm that might destabilize the tRNA_{Leu} structures and target them to rapid degradation.

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(ii) The second group contained tRNA\textsuperscript{Leu} mutations inducing decreases of the \textit{in vivo} aminoacylation levels: G2U, G30U, A35G, G36U and G37U. The G2U mutation probably destabilized the structure of the acceptor arm by changing the G:U wobble base pair to a U–U. G2U mutation caused a decrease in the aminoacylation level and a decrease in growth of the yeast cells. Due to its location, G2U might influence the CCA acceptor end conformation during the aminoacylation reaction and explain the decrease of aminoacylation level. The decrease of the aminoacylation level of mutant G30U is even lower compared to G2U, however, it impacts growth rate of yeast cells as G2U mutant. Therefore, this moderate effect can hardly explain the \textit{in vivo} growth defect. As G30U introduced a U\textsubscript{30}–C\textsubscript{40} mismatch in the anticodon-arm, one might speculate that the mutation impacts protein biosynthesis after aminoacylation, during codon reading on the ribosome, for instance.

Yeast cells harbor four distinct tRNA\textsuperscript{Leu} isoacceptors decoding the six Leu codons. The variety of anticodons suggests that the anticodon triplet is not crucial for LeuRS recognition. Indeed, our data showed that A35G and G36U mutation caused only moderate decreases of the aminoacylation levels of \textasciitilde40\% and 20\%, respectively. This indicates that nucleotides from the anticodon loop are involved in some way in the aminoacylation process as previously proposed (34). However, in our case, the two mutations were lethal because they induced a loss of Leu-decoding capacity of the yeast cells and not because of the reduced aminoacylation.

Nucleotide 37 was also isolated as a lethal mutation G37U. Nucleotide 37, which is often a modified nucleotide (54), is involved in the reading frame maintenance, ensuring accurate translation on the ribosome (42,54). For instance, a previously isolated G37U mutation in yeast tRNA\textsuperscript{Tyr} was shown to be lethal despite normal transcription and aminoacylation levels (55), suggesting a defect at the translation level. Similarly, mammalian tRNA\textsuperscript{lys} lacking A37 modification caused type II diabetes (56). Surprisingly, our data showed that G37U mutation in yeast tRNA\textsuperscript{Leu} totally abolished aminoacylation \textit{in vivo}. We further purified G37U mutated tRNA from yeast and found that its aminoacylation was also abolished \textit{in vitro}. Therefore, the lethal effect was first due to a defect of aminoacylation and revealed that G37 is an essential determinant for LeuRS recognition.

(iii) The third group of mutations contained G42U and derivative G42U/C28A, both located in the anticodon arm. Both induced a growth inhibition when the yeast cells were grown on medium containing high amounts of Nva, an analog of Leu, which is usually edited by LeuRS to prevent its incorporation into proteins. Editing of tRNA by LeuRS is dependent on the translocation of the 3′ CCA end from the synthetic site to the editing site (8,57). Different nucleotides from the tRNA are involved in the process. In \textit{E. coli} tRNA\textsuperscript{Val}, a mutation of A76U specifically abolished the editing response without impairing the aminoacylation (58). In tRNA\textsuperscript{Leu} from \textit{A. aeolicus}, discrete editing determinants in the anticodon arm were found to stabilize the conformation of the post-transfer editing state. In the present work, we identified a G42U mutation that reduced tolerance of yeast to excess of Nva, suggesting some toxic effect due to decrease of the editing activity of LeuRS. The data also showed that a strong C\textsubscript{28}–G\textsubscript{42} base pair is strictly required and cannot be replaced by a A:U pair. In both cases, the aminoacylation of tRNA\textsuperscript{Leu} was kept intact. Here the data show that yeast growth was considerably reduced despite standard aminoacylation and tRNA levels. This strongly suggests that the toxic effect was due to the inability to clear mischarged products that appeared in the presence of elevated concentrations of Nva. This hypothesis was further confirmed by showing that the editing activity of purified G42U mutant was reduced, leading to Nva-adenylate accumulation that could be later charged on tRNA\textsuperscript{Leu} and incorporated into proteins.

tRNA\textsuperscript{Leu}(UAG) triple knockout strain as a tool to obtain tRNA\textsuperscript{Leu} mutants

Transcripts from \textit{S. cerevisiae} tRNA\textsuperscript{Leu} (both GAG and UAG isoacceptors) are very poor substrates for LeuRS and hardly used for biochemical investigations (15). The study on the interaction between yeast tRNA\textsuperscript{Leu} and LeuRS is difficult. We could not obtain yeast tRNA\textsuperscript{Leu} with high enough accepting activity from \textit{E. coli} transformants containing its gene or by T7 polymerase transcription. Using the knockout strains \textit{tl(uag)-1-3}, we could not only investigate the function of tRNA\textsuperscript{Leu} by the growth rate of yeast cells \textit{in vivo} but also obtain any tRNA\textsuperscript{Leu}(UAG) mutant with the modified bases from yeast \textit{tl(uag)-1-3} transformants containing the mutant’s gene. \textit{In vitro} the studies on effect of a series of interesting individual nucleotide of yeast tRNA\textsuperscript{Leu}(UAG) on its function becomes possible. We will use the yeast knockout strains to study the effect of more nucleotides of tRNA\textsuperscript{Leu}(UAG) on aminoacylation and editing of \textit{ycLeuRS} by site-directed mutagenesis. Further studies are in progress to find new selection tools and perform more extensive analysis of the tRNA properties.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–5.

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