

A yeast knockout strain to discriminate between active and inactive tRNA molecules

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ABSTRACT

Here we report the construction of a yeast genetic screen designed to identify essential residues in tRNA^{Arg}. The system consists of a tRNA^{Arg} knockout strain and a set of vectors designed to rescue and select for variants of tRNA^{Arg}. By plasmid shuffling we selected inactive tRNA mutants that were further analyzed by northern blotting. The mutational analysis focused on the tRNA D and anticodon loops that contact the aminoacyl-tRNA synthetase. The anticodon triplet was excluded from the analysis because of its role in decoding the Arg codons. Most of the inactivating mutations are residues involved in tertiary interactions. These mutations had dramatic effects on tRNA^{Arg} abundance. Other inactivating mutations were located in the anticodon loop, where they did not affect transcription and aminoacylation but probably altered interaction with the translation machinery. No lethal effects were observed when residues 16, 20 and 38 were individually mutated, despite the fact that they are involved in sequence-specific interactions with the aminoacyl-tRNA synthetase. However, the steady-state levels of the aminoacylated forms of U20A and U20G were decreased by a factor of 3.5-fold *in vivo*. This suggests that, unlike in the *Escherichia coli* tRNA^{Arg}:ArgRS system where residue 20 (A) is a major identity element, in yeast this position is of limited consequence.

INTRODUCTION

Mutant tRNAs have been widely used to study the mechanism of recognition between tRNAs and the corresponding aminoacyl-tRNA synthetases (aaRS). The 1990s were the years of *in vitro* studies that involved T7 RNA polymerase transcription of mutant tRNA genes. This powerful approach allows the synthesis of all the desired mutations at any position. However, due to the large number of possible mutations, this approach is time consuming and costly.

Moreover, the absence of base modifications in the T7 RNA polymerase products can act negatively or positively on aaRS recognition (1–3) or modify the physico-chemical properties of these molecules and thus the assay conditions (4). *In vivo* approaches have been less frequently used. Amber, ochre and opal tRNA suppressors are efficient tools to monitor the real *in vivo* amino acid acceptance, but they suffer from sequence constraints at the anticodon level (5–7). Efficient tools were developed to monitor and analyze the *in vivo* amino acid acceptance of these suppressors, such as incorporation in a reporter protein followed by N-terminal sequencing (5) or northern blot analysis under acidic conditions (8). This last method is particularly efficient to measure the *in vivo* aminoacylation level and verify the tRNA identity. More recently, tRNA knockout strains have been used as a selection tool to study the structure–function relationships of tRNAs *in vivo* (9–11).

Actually, a powerful approach to study the critical nucleotides of tRNA would require the rapid preparation of large libraries of mutated RNAs and an *in vivo* screen of the libraries in order to select tRNA genes for the desired phenotype. A typical example of such a study was described recently in which a combinatorial tRNA library was built and was screened in a tRNA^{Ala} knockout strain (12).

Here we describe the search for critical residues of yeast tRNA^{Arg4}_{CCG}, one of the two minor isoacceptors of the arginine system. Previous structural investigations have shown that yeast ArgRS interacts with the major form tRNA^{Arg2}_{ICG} in a canonical class I manner. The enzyme contacts the tRNA on the D loop side and interacts with the major groove of the acceptor stem and anticodon loop. The direct contacts between the two partners involve the terminal adenosine with the catalytic site, several residues of the D loop and some residues of the anticodon loop (13). In terms of amino acid identity, residues that confer arginine acceptance are C35 and G36 or U36, as shown by kinetic analyses with *in vitro* synthesized transcripts (14).

In the present work we used an *in vivo* approach in order to identify the critical residues of tRNA^{Arg} that interact with ArgRS. First, we created a knockout strain of the minor tRNA^{Arg4}_{CCG} isoacceptor by disrupting its single gene in *Saccharomyces cerevisiae*. Then, a randomly mutated library of the same tRNA was generated and tested by plasmid

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shuffling in the knockout strain. In this way, 17 lethal mutants were isolated and were further analyzed for their tRNA content by northern blotting under acidic conditions. To understand the function of several non-essential interactions that were observed in the crystal structure of ArgRS:tRNA^{Arg}, we also analyzed the RNA contents of five viable mutants. Among them, the U20A and U20G mutants exhibited a 3.5-fold decrease in their steady-state levels of aminoacylation, demonstrating that the yeast cells can survive and grow with reduced amounts of aminoacylated tRNA.

MATERIALS AND METHODS

Enzymes, chemicals and oligonucleotides

Restriction enzymes, T7 DNA polymerase, bacterial alkaline phosphatase and polynucleotide kinase were from New England Biolabs (USA). [α -³⁵S]dATP, [γ -³²P]dATP, [¹⁴C]L-arginine and Hybond-XL nylon membrane were from Amersham Pharmacia (UK). 5-Fluoroorotic acid (5-FOA) was from Toronto Research Chemicals (Canada). Oligonucleotides were synthesized by NAPS (Germany) and Genset (France).

Vectors, bacterial and yeast strains

Escherichia coli TB1 [F⁻ *ara* Δ (*lac-proAB*) *hsdR* (rk⁻ mk⁺) *rpsL*(Str^r) [ϕ 80, *dlac* Δ (*lacZ*)M15] was used as a recipient for cloning procedures. *Saccharomyces cerevisiae* YBAM2 2n (*ura3-52 lys2-801^{am} trp1- Δ 63 his3- Δ 200 leu2- Δ 1 ade2- Δ 450 ade3- Δ 1483*) was used for the disruption of the tRNA^{Arg4} gene. The resulting strain, *S.cerevisiae* YAL5 [YBAM2, *tr4::HIS3*, pAL5 (tR4⁺, Ura⁺, Ade3⁺)] was used to assay the mutant phenotypes and to extract RNA for northern blot analysis. This strain carries a knockout of the tRNA^{Arg4}_{CCG} gene (*tR4*) and is rescued by a plasmid (pAL5) carrying the native gene. The bacterial and yeast strains were grown and transformed according to standard procedures.

A 0.65 kb PCR fragment containing the tRNA^{Arg4} gene was cloned into several plasmids: pRS315-*tR4* (tR4⁺, Leu⁺), pRS314-*tR4* (tR4⁺, Trp⁺), pAL5 (tR4⁺, Ura⁺, Ade3⁺) a derivative of pALR10 (15). pRS315-*tR3* was constructed by cloning a 0.4 kb HindIII fragment encoding the native tRNA^{Arg3}_{mcm5UCU} gene (16). This vector was used to generate the pRS315-*tR3*_{CCG} vector by site-directed mutagenesis; this vector is Leu⁺ and codes for a chimeric tRNA^{Arg3} with a tRNA^{Arg4} anticodon (tRNA^{Arg3}_{CCG}).

In situ disruption of the gene encoding tRNA^{Arg4}

The 1.8 kb fragment encoding the *His3* gene was inserted in the NsiI site of the tRNA^{Arg4}_{CCG} gene located on the pRS315-*tR4* vector. This new vector served as a source for the 2.45 kb DNA fragment containing the disrupted *tR4* gene. By double homologous recombination the *tR4* gene of the diploid yeast strain YBAM2 was disrupted (17). His⁺ colonies were selected and were induced to form spores on sporulation plates. Then, tetrads were dissected with a Singer apparatus and haploids were plated on YPD plates. Only two His⁻ viable spores were expected in the case of an essential gene. Then, to generate the haploid strain YAL5, disrupted for *tR4* and rescued by a plasmid copy of *tR4*, the diploid colonies showing 2:2 segregation were transformed by plasmid pAL5 (tR4⁺, Ura⁺,

Ade3⁺) and sporulation was again induced. Among the four viable spores, two gave the expected His⁺, red and 5-FOA^s phenotype corresponding to the haploid strain designated YAL5. The integration event was further confirmed by PCR analysis of the genomic DNA. The sect⁺ character, resulting from the introduction of another plasmid *tR4* gene, was tested after introduction *in trans* of pRS314-*tR4*.

Random mutagenesis and *in vivo* selection

A 32 nt long deoxyoligonucleotide was used to generate a library containing randomly mutated *tR4* genes (18). This primer was designed in order to generate statistically one mutation per *tR4* gene. It had the sequence GTGCA ATGGT TAGCA TGCAT TCTTC CGGTG GC. The underlined positions contained a mixture of 90% of the wild-type nucleotide and 10% of a mixture of the three other nucleotides. This primer was complementary to positions 10–41 of *tR4*.

An aliquot of 10 ng of the pRS314-*tR4* library was used to transform the YAL5 strain on minimal medium supplemented with adenine (limiting concentration 2 μ g ml⁻¹), uracil (20 μ g ml⁻¹), lysine (20 μ g ml⁻¹) and leucine (60 μ g ml⁻¹). The concentration of 10 ng was determined in order to obtain 50–100 colonies per 88 mm diameter Petri dish. After 72 h incubation at 30°C, the Trp⁺ red (Sec⁻) colonies were isolated and screened for 5-FOA resistance. The Trp⁺ 5-FOA sensitive (5-FOA^s) colonies were selected and were designated as containing an inactive *tR4* gene on the pRS314 vector.

Site-directed mutagenesis

To complete the first round of *in vivo* selection, site-directed mutagenesis was performed with 23 different primers by the Kunkel procedure (18). The primer sequences were as follows (mutated positions in bold).

U16C, CTAGT GCAAC GGTTA GCATG; U16A, CTAGT GCAAA GGTTA GCATG; U16G, CTAGT GCAAG GGTTA GCATG; U20A, GTGCA ATGGA TAGCA TGC; U20C, GTGCA ATGGC TAGCA TGC; U20G, GTGCA ATGGG TAGCA TGC; U20Aa, GCAAT GGTA GCAATGC; U20Ca, GCAAT GGTC GCAATGC; U20Ga, GCAAT GGTGA GCAATGC; A21C, GCAAT GGTT CCAATGC; A21G, GCAAT GGTTG CCAATGC; A21T, GCAAT GGTTT CCAATGC; U32A, GCATG CATT CCAATGC; U32C, GCATG CATT CCAATGC; U32G, GCATG CATT CCAATGC; U33A, CATGC ATTCT ACCGG TGGC; U33C, CATGC ATTCT CCCGG TGGC; U33G, CATGC ATTCT GCCGG TGGC; G37A, GCATT CTCC GATGG CTGTG ATCC; G37C, GCATT CTCC GCTGG CTGTG ATCC; U38A, CATT CTTCC GAGGC TGTGA TCC; U38C, CATT CTTCC GCGGC TGTGA TCC; U38G, CATT CTTCC GGGGC TGTGA TCC.

Analysis of *in vivo* aminoacylation

Yeast clones carrying mutated *tR4* genes were cured of the rescuing pAL5 plasmid (tR4⁺, Ura⁺, Ade3⁺). For this, the mutated clones were transformed by pRS315-*tR3*_{CCG} and plated on minimal medium supplemented with adenine (limiting concentration 2 μ g ml⁻¹), uracil (20 μ g ml⁻¹) and lysine (20 μ g ml⁻¹). After incubation, red/white sector colonies were observed. White sectors were isolated since they correspond to cells cured of pAL5: YAL5 containing

pRS314-*tR4* and pRS315-*tR3_{CCG}*. An aliquot of 100 ml of the same liquid medium was inoculated and grown to an A_{700} of 2, then cells were harvested by centrifugation. All following steps were performed on ice. Cell pellets were resuspended in 5 ml of extraction buffer (0.3 M sodium acetate, pH 4.5, 10 mM EDTA) and subjected to two phenol extractions (pH 4.5). After ethanol precipitation the RNA samples were dissolved in 200 μ l of extraction buffer and quantified by absorbance measurement at 260 nm.

A sample of 30 μ g of total RNA was subjected to northern blot analysis. Aliquots of 15 μ l were supplemented with 5 μ l of loading buffer (0.1 M sodium acetate, pH 4.5, 8 M urea, 0.2% bromophenol blue and 0.2% xylene cyanol) and separated on a 1 mm thick 6.5% polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing 8 M urea in 0.1 M sodium acetate buffer pH 5. Electrophoresis was performed at 18 W (~400 V) at 4°C for 24 h. The portion of the gel between the two dyes was transferred and baked at 80°C to a nylon membrane (Hybond-XL) using a gel dryer. Prehybridization was performed for 4 h at 60°C in a 40 ml solution (1 \times Denhardt's solution, 5 \times SSPE, 0.5% SDS). Hybridization was performed for 12 h at 60°C in 15 ml of the same solution, in the presence of a tRNA^{Arg} probe, a 5S probe (as an internal control) and a tRNA^{Leu}_{UAA} probe (to control the aminoacylation level of the samples). Each probe was 5' end ³²P-labeled. The probes were: tRNA^{Arg4}_{CCG}, 5'-CTCGA ACCCG GATCA CAGCC, annealing to positions 39–59; 5S, 5'-ACCCA CTACA CTACT CGGTC AGGCT CTTAC; tRNA^{Leu}_{UAA}, 5'-GGATG CGAGG TTCGA ACTCG CGCGG. Washing was performed in 2 \times SSPE, 0.5% SDS solution for 20 min at 50°C. Signals were quantified using a Fuji Bioimager Bas2000.

Transcription with T7 RNA polymerase and aminoacylation

The gene for tRNA^{Arg4}_{CCG} and the different mutants were constructed with four DNA oligonucleotides that were ligated between the HindIII and BamHI restriction sites of plasmid pUC18. Transcription using T7 RNA polymerase was performed according to standard procedures (19). After phenol extraction and ethanol precipitation, transcripts were purified using denaturing PAGE. Full-length tRNAs were eluted from gel slices using a Schleicher & Schüll (Dassel, Germany) electroelution apparatus.

Aminoacylation assays were carried out at 37°C in 50 mM HEPES (pH 7.5), 30 mM KCl, 25 μ M [¹⁴C]L-arginine (25–300 Ci mol⁻¹), 10 mM ATP, 10 mM MgCl₂, 0.1 mg ml⁻¹ bovine serum albumin, 5 μ M tRNA transcript and 30 nM ArgRS, as described previously (15).

RESULTS

Gene cloning and *in situ* disruption

The yeast genome contains 19 tRNA^{Arg} genes distributed as six tRNA^{Arg2}_{ACG} genes, eleven tRNA^{Arg3}_{UCU} genes and two unique genes for tRNA^{Arg1}_{CCU} and tRNA^{Arg4}_{CCG} each. We chose to disrupt the tRNA^{Arg4}_{CCG} gene, whose decoding capacity is not redundant with the other tRNA^{Arg}. The tRNA^{Arg4}_{CCG} gene is located on chromosome XII and is flanked by ORFs YLR343w and YLR344w. By PCR

amplification we isolated a 647 bp HindIII fragment which was cloned in the same site of pRS315 (15). The tRNA gene was disrupted by inserting a 1.8 kb DNA fragment encoding the *HIS3* gene. Finally, this DNA fragment encoding the tRNA gene disrupted by *HIS3* (*tr4::HIS3*) was excised from the pRS315 vector and used to replace the wild-type copy of the tRNA gene in the yeast strain YAMB4. The tetrads resulting from meiosis and sporulation were dissected: two spores were unable to grow whereas the two growing spores were His⁻. This was the first evidence showing that the tRNA^{Arg4}_{CCG} gene is an essential gene. When transformed with a rescuing plasmid encoding the tRNA^{Arg4}_{CCG} gene (pAL5: Ura⁺, Ade3⁺, tRNA^{Arg4}_{CCG} gene) the disrupted diploid gave four viable spores after segregation. Two of them were His⁻ and were able to grow on 5-FOA medium, whereas the other two were His⁺ and 5-FOA^s, demonstrating that, when disrupted for the tRNA^{Arg4} gene, yeast cells cannot lose the rescuing plasmid encoding the tRNA gene. We conclude that the tRNA^{Arg4}_{CCG} gene is an essential gene. The resulting disrupted strain was called YAL5.

Construction of a genetic screen based on the knockout strain

tR4 is an essential gene, as shown by the inability of spores that are disrupted for *tR4* to grow. By introducing a copy of *tR4* [on pAL5 (tR4⁺, Ura⁺, Ade3⁺)] before meiosis we can rescue the mutant spores and thus generate haploid strains disrupted for *tR4* and harboring a plasmid copy of *tR4*. We used this property to design a genetic approach for selection of tRNA^{Arg4}_{CCG} mutants affected in their functions (Fig. 1). For the recipient yeast cell, a genetic background (Ade2⁻, Ade3⁻) was chosen in order to give a red pigmentation when rescued by the Ade3⁺ vector (pAL5) and grown on medium containing limiting amounts of adenine (see Materials and Methods). Moreover, the presence of *URA3* in this vector confers sensitivity to 5-FOA on the transformed cells. The screening procedure is based on the exchange of *tR4* carried by pAL5 for another *tR4* carried by a pRS314 vector (tR4⁺, Trp⁺). This exchange can occur when the second *tR4* encodes a native tRNA and when the cells are spread on suitable growth medium. The exchange of pAL5 by pRS314-*tR4* is followed by a loss of red pigmentation (colonies appear white/red sector, sect⁺), due to *ADE3* loss, and by 5-FOA resistance, due to *URA3* loss. Using this approach, it becomes possible to select from a library of mutated *tR4* genes those coding for an inactive tRNA^{Arg4}_{CCG}. These clones will appear red and non-sector (red, sect⁻ and 5-FOA^s) since the rescuing pAL5 is essential for viability and cannot leave the transformed cell. Thus, this convenient selection for lethal *tR4* is based on visual discrimination of the colonies.

Analysis of the selected random mutations

By the shuffle screen described above, we isolated 5000 colonies co-transformed with pAL5 and with the mutated library pRS314-*tR4*. We found 33 red, sect⁻ colonies that were unable to lose the rescuing pAL5 vector. From these clones we extracted the pRS314-*tR4* plasmids and confirmed the red sect⁻ phenotype by re-introducing them into the YAL5 strain. Then the *tR4* alleles and flanking regions were sequenced. Twenty-nine single substitutions, one single nucleotide deletion and three double mutants were identified. The mutants

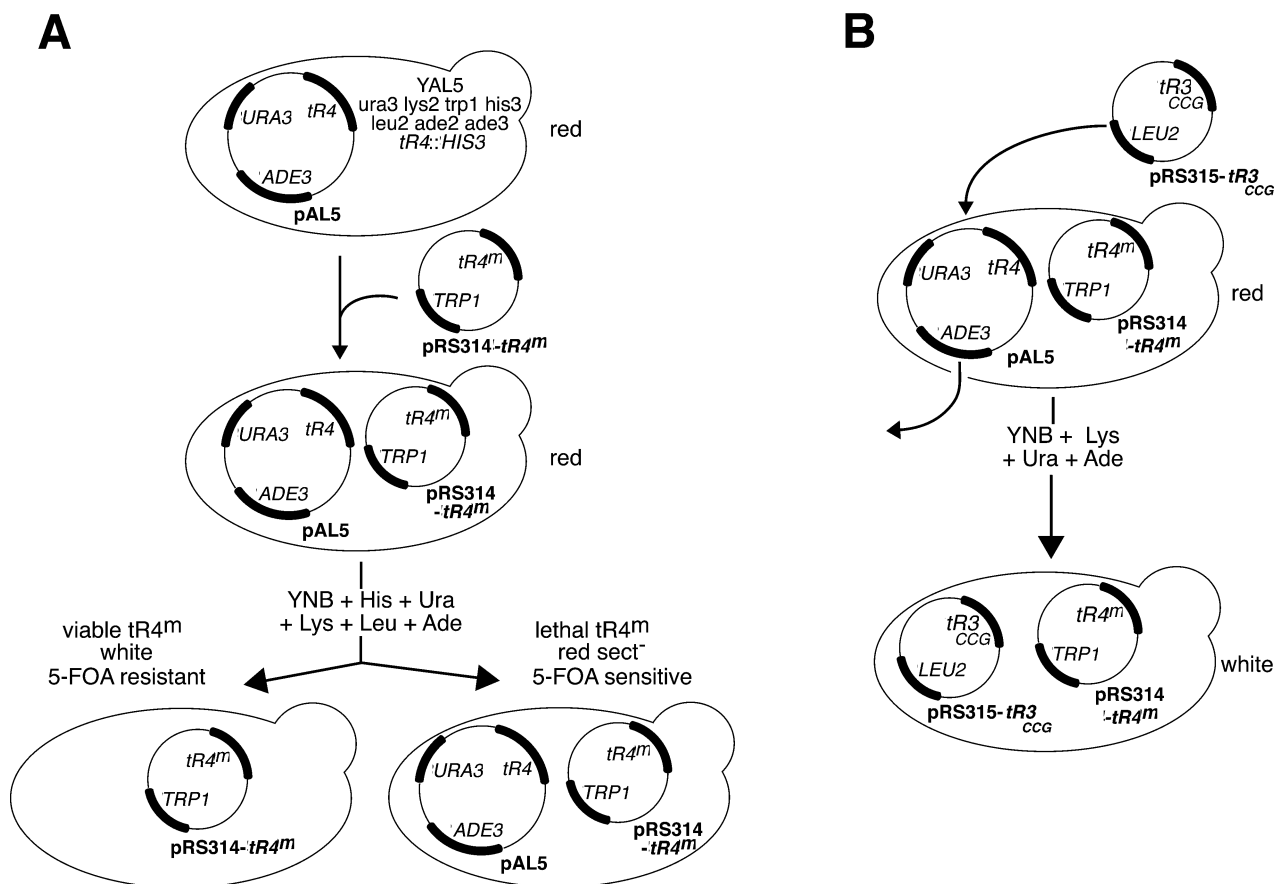


Figure 1. Schematic representation of the plasmid shuffle procedures used for the selection and analysis of tRNA^{Arg4} mutated genes. (A) Selection of lethal tRNA^{Arg4} genes. The *tR4*-disrupted strain YAL5 (*tr4::HIS3*), rescued by pAL5 (*tR4*, *ADE3*, *URA3*), was transformed by the mutated *tR4* library (pRS314-*tR4*^m). The resulting yeast strain was red according to the Ade3⁺ Ade2⁻ phenotype. In the presence of a functional tRNA^{Arg4} encoded by pRS314-*tR4*^m, pAL5 was lost and the resulting strain became Ade3⁻ Ade2⁻ (white or white/red sectored colonies). In the presence of an inactive *tR4*^m (lethal *tR4*), pAL5 could not be lost and the colonies remained non-sectored red (red sect⁻ colonies). (B) To analyze the aminoacylation levels of tRNA^{Arg4}_{CCG} by northern blot the rescuing native *tR4* gene from pAL5 (*tR4*, *ADE3*, *URA3*) was shuffled from the red strains by transformation with pRS315-*tR3*_{CCG} (*tR3*_{CCG}, *LEU2*). In this way, the northern blot signal measured with the tRNA^{Arg4}_{CCG} probe was specific to the mutated *tR4* alleles.

appeared one or more times: A14C, one occurrence; A14G, one occurrence; A14U, four occurrences; A15C, two occurrences; A15G, two occurrences; A15U, six occurrences; G18A, one occurrence; G18C, two occurrences; G18U, four occurrences; G19A, two occurrences; G19C, one occurrence; G19U, two occurrences; G37U, one occurrence; ΔA15, one occurrence; A14U/A15G, two occurrences; A15C/G19U, one occurrence. In fact, only six different positions in tRNA^{Arg} were selected as lethal mutants, whereas 12 different positions statistically were degenerate in the oligonucleotide used for the mutagenesis. Lethal mutations affected mainly nucleotides of the D loop, particularly those involved in tertiary and triple interactions. Only one mutation of position 37 of the anticodon loop was found to have a lethal effect. Despite repetitive screens, no different lethal mutants could be isolated from the randomly mutated library. To ensure that the library is complete and exhaustively screened we sequenced 48 clones of the library that confirmed the random distribution of the mutations. Nevertheless, in order to strictly screen the 12 selected positions we constructed the missing substitutions by site-directed mutagenesis and assayed them *in vivo*.

Site-directed mutagenesis to complete the first set of identified essential nucleotides

Twenty-three new mutants were built by site-directed mutagenesis. In the shuffle assay 19 mutants were viable (sect⁺, 5-FOA^r), but only four were lethal (red, sect⁻, 5-FOA^s); these were A21G and A21U in the D loop and U33G and U33A in the anticodon loop. Thus, the *in vivo* selection was very efficient, since only four mutants escaped the first selection procedure.

In summary, 17 lethal mutations were selected among the 36 mutations generated at 12 positions. Positions 14, 15, 18 and 19 did not accept any other nucleotide than the native one. In contrast, positions 21, 33 and 37 can tolerate some mutations (Fig. 2).

Northern blot analysis under acidic conditions

Lethal mutations can inactivate the tRNA function at different levels. Transcription, processing, stability, modification, aminoacylation and interaction with the translation factors and the ribosome are steps that can be crucially affected by

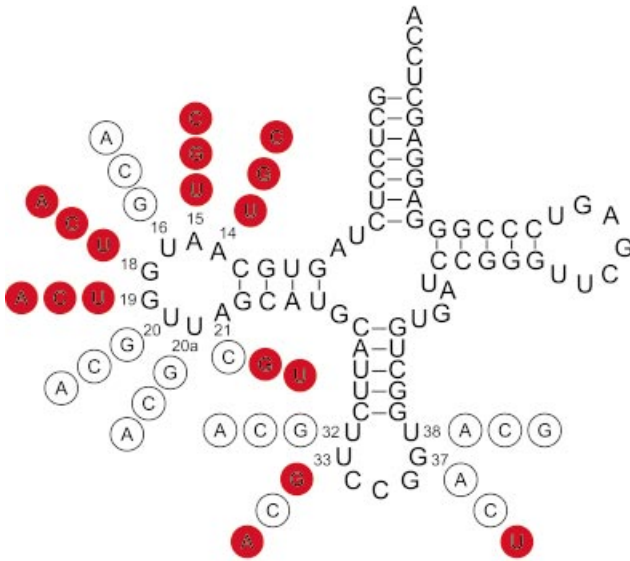


Figure 2. Secondary structure of *S. cerevisiae* tRNA^{Arg4}_{CCG}. Numbering is according to Sprinzl *et al.* (32). The encircled nucleotides are the mutations found at corresponding positions. Red filled circles indicate the lethal mutations that gave inactive tRNAs *in vivo*. They exhibited a non-sectored red phenotype. Empty circles contained nucleotides that gave active tRNAs *in vivo* (red-white sectored phenotype).

single point mutations. Northern blot analysis is a powerful method to investigate the global effect of mutations *in vivo*. Extraction of RNA under acidic conditions and acid gel separation allows the measurement of the aminoacylation levels. Acid conditions preserve the ester bond between the tRNA and the amino acid and thus aminoacylated tRNA can be separated from uncharged tRNA and visualized by hybridization with a specific probe (8). In the case of lethal mutations, the yeast strain contains, in addition to the mutated tRNA^{Arg4}_{CCG} (from pRS314-*tR4*), the rescuing native tRNA^{Arg4}_{CCG} (from pAL5), which might also hybridize with the labeled probe. To distinguish between the two species an

appropriate choice of oligonucleotide and appropriate washing temperatures can be used. However, this makes the comparison between the different mutants difficult. To circumvent this problem we substituted the rescuing native tRNA^{Arg4}_{CCG} by a tRNA^{Arg3}_{CCG} which is able to read the codon CGG usually read by tRNA^{Arg4}. In this way, only the lethal tRNA^{Arg4}_{CCG} from pRS314-*tR4* will be detected by northern blot assay, with a single probe and annealing protocol for all mutants. tRNA^{Arg3}_{CCG} was engineered by site-directed mutagenesis and cloned into pRS315 (Leu⁺). Lethal clones were transformed by pRS315-*tR3*_{CCG} (tR3_{CCG}⁺, Leu⁺) and shuffled for the rescuing plasmid pAL5 (tR4⁺, Ura⁺, Ade3⁺) on Ura-containing medium (Fig. 1).

Northern blot analysis revealed the absence of mutated tRNA^{Arg4} in most of the clones exhibiting a lethal phenotype (Fig. 3). Only four mutants exhibited a normal level of mutated tRNA (G19C, U33A, U33G and G37U), with a surprising normal level of aminoacylation, despite their lethal phenotype. The genes for these mutants were transcribed by T7 RNA polymerase and were assayed for their aminoacylation properties (data not shown). They were aminoacylated with parameters comparable to the native one except that the K_m value of mutant G19C was increased 14-fold ($K_m = 1.4 \mu\text{M}$). We also analyzed the content of aminoacyl-tRNA in several viable mutants for which an effect on the aminoacylation efficiency was suspected. These were mutants at positions 16, 20 and 38. The complete set of mutants was assayed for position 20 (U20A, U20C and U20G) and a unique transversion mutation was tested for each of the other positions (U16A and A38U). The expression level of the four mutants was comparable to the wild-type level, but a significant decrease in the charging level was observed for mutants U20A and U20G (22 and 20% aminoacylation, respectively, compared to the 69% of wild-type tRNA^{Arg4}) (Fig. 4). Moreover, mutant U20A migrates faster than the other tRNA, suggesting an incomplete denaturation of its tertiary structure in the low temperature conditions used to run the acid urea gels. Another reason for the fast mobility could be a change in the modified base content of this tRNA.

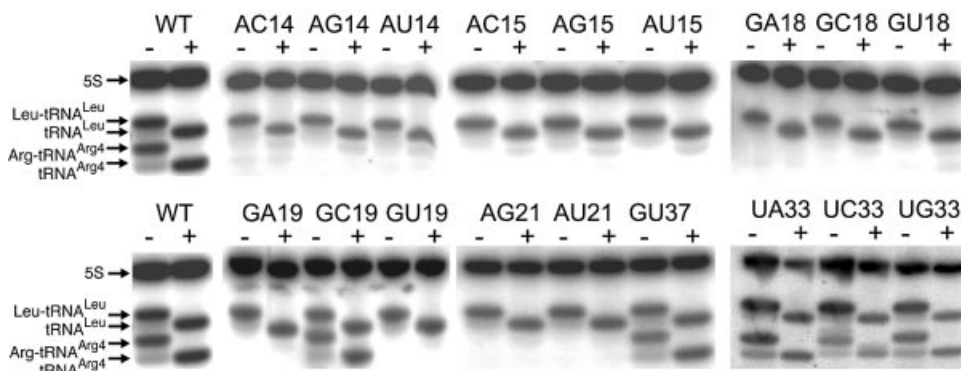


Figure 3. Northern blot analysis of tRNA samples electrophoresed on a 6.5% polyacrylamide gel containing 8 M urea at pH 5.0 and 4°C. Total RNA from lethal mutants was extracted under acidic conditions, migrated and transferred to a Hybond membrane. The blot was probed with a mixture of ³²P-labeled oligonucleotides complementary to 5S RNA (control for RNA content), to tRNA^{Leu} (control for the acidic extraction procedure) and to tRNA^{Arg4}_{CCG}. + and - marks refer to alkaline treatment used to deacylate the RNA. Most of the extracts from mutants of the D loop are deprived of tRNA^{Arg4}_{CCG}, in contrast to those of the anticodon loop that are expressed and aminoacylated. Except for U33C, all the RNA samples are from lethal mutants.

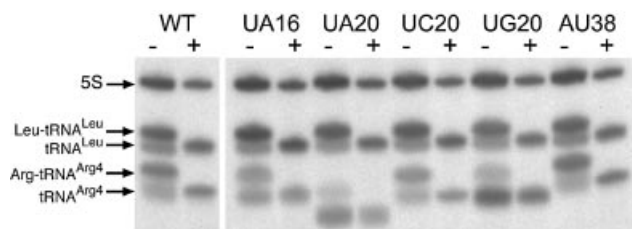


Figure 4. Northern blot analysis of tRNA samples separated on a 6.5% polyacrylamide gel containing 8 M urea at pH 5.0 and 4°C. + and – refer to alkaline treatment used to deacylate the RNA. The three U20 mutants and one representative U16 and A38 mutant were analyzed. U20A and U20G were only charged up to 22 and 20%, respectively, compared to 69% for the wild-type tRNA.

DISCUSSION

In vivo approaches are useful to screen for genes carrying a desired phenotype among large libraries of degenerate genes. tRNA suppressors have been widely used for studies of aaRSs recognition of their cognate tRNA. But they suffer from the presence of non-cognate anticodon nucleotides that sometimes strongly determine another identity. Therefore, many tRNA suppressors are glutamine or lysine inserting suppressors (5), a phenomenon that singularly reduces the interest of these studies.

As tRNAs are essential for the cell, it was tempting to use their genes as genetic tools to analyze tRNA function. However, tRNA genes are small and are often present as several copies, which are two major difficulties to create knockout strains or to isolate conditional strains. For these reasons, only a few strains containing mutated chromosomal tRNA genes have been described. A thermosensitive *E. coli* tRNA^{Trp} mutant was described, because of its unique copy in the bacterial genome (20). This strain was later used to study the identity elements of *E. coli* tRNA^{Trp} (21). More recently, two *E. coli* knockout strains were engineered for tRNA^{Ala} and tRNA^{Asp} (9,11). They required inactivation of three tRNA genes in each case and the resulting bacterial strains were rescued by plasmid copies of the corresponding tRNAs.

In this work we chose the arginine system of *S. cerevisiae* to build a new tRNA knockout strain. In the genome of *S. cerevisiae*, 19 tRNA^{Arg} gene copies are present (22). Four distinct isoacceptors are found that decode the six different Arg codons. Two tRNAs, tRNA^{Arg2}_{ICG} and tRNA^{Arg3}_{mcm5UCU}, are preponderantly found in yeast cells where they read CG(C/U/A) and AG(G/A) codons, respectively. They are encoded by 6 and 11 gene copies, respectively. A third isoacceptor is tRNA^{Arg1}_{CCU}, which reads the AGG codon. It is encoded by a single gene copy and is dispensable, as shown by the viable phenotype of the knockout strain (23). Indeed, AGG codons can be read by tRNA^{Arg2}_{ICG}. The last tRNA is the minor tRNA^{Arg4}_{CCG}, which reads the rarest Arg codon CGG (3.9% of the Arg codons) and which is encoded by a single gene copy. By disrupting the gene encoding tRNA^{Arg4}_{CCG} we thought we could selectively impair reading of the 9827 CGG codons present in the yeast genome and thus have a lethal phenotype that could be rescued by a plasmid copy of the tRNA gene. The resulting *tr4::HIS3* disruptant was lethal, indicating that tRNA^{Arg4}_{CCG} is the sole tRNA species decoding the CGG

codon. The disrupted strain was rescued by a plasmid-encoded tRNA^{Arg4}_{CCG} and a shuffle method was used to screen a library of mutated copies of *tR4*. Thanks to colored yeast genetic markers, it was possible to visually analyze the plasmid shuffling and thus discriminate between the viable and the lethal mutated *tR4* genes. We focused the mutational analysis on the tRNA parts that are single stranded and in close vicinity to the synthetase in the complex, namely the D loop and the anticodon loop. We deliberately excluded the double-stranded regions of the stems since mutations here easily generate lethality by destabilizing effects on the tRNA structure. Also, the anticodon triplet was excluded from our mutational analysis, since it cannot be modified without changing the decoding properties of the tRNA.

Specific contacts between ArgRS and tRNA residues 16, 20 and 38 are not essential

Next to the anticodon triplet, three other residues of tRNA^{Arg2}_{ICG} interact specifically with ArgRS (13). These are residues 16 and 20 of the D loop and residue 38 next to the anticodon bases. Oxygen atoms of the U16 ring interact with Lys543 (Nζ), Trp60 (N) and Arg36 (NH2). U20 (O2, N3, O4) atoms interact with Asn106 (Oδ1) and Gln111 (Ne2), whereas A38 (N6) interacts with the terminal carboxyl group of the protein (Met607-OT2) (13).

Among the three residues, residue 20 deserves most attention. In most organisms residue 20 is a well-conserved adenine that crucially contributes to the arginine identity, as shown by studies on *E. coli* (24–27). However, fungi like *S. cerevisiae* or *Schizosaccharomyces pombe* and *Neurospora crassa* and some mitochondrial tRNA^{Arg} contain C or D instead of A at position 20. In tRNA^{Arg3}_{UCU} from *S. cerevisiae* it was shown that this residue does not play a role in the arginine identity but acts as an antideterminant against *E. coli* ArgRS (28). The *in vivo* study on tRNA^{Arg4}_{CCG} performed here confirms the dispensable character of this interaction with the synthetase. The three mutants at position 20 were able to complement the tRNA^{Arg4} knockout strain, suggesting that this interaction is a simple remnant of evolution. Nevertheless, the analysis of the *in vivo* aminoacylation levels of these tRNA species showed a 3.5-fold drop for the two transversion mutations U20A and U20G (Fig. 4). This shows that the interactions that occur with U20 are not simple relics but still play an active role in the aminoacylation of tRNA^{Arg4}_{CCG}. Moreover, the results show that yeast cells can grow with a reduced amount of acylated tRNA, whereas it is usually believed that *in vivo* 100% of the tRNA molecules are in the acylated form and are in complex with eEF1A. In fact, it seems that in the arginine system the *in vivo* aminoacylation level never reaches 100% but only 69% for tRNA^{Arg4}_{CCG}, 68% for tRNA^{Arg1}_{CCU}, 71% for tRNA^{Arg2}_{ICG} and 74% for tRNA^{Arg3}_{mcm5UCU} in different genetic backgrounds (data not shown). For now, we can only speculate on the reason for this partial *in vivo* aminoacylation. A low stability of the arginyl-ester bond might be the simplest explanation for a decreased level of aminoacylated tRNA^{Arg}. Another reason might be that ArgRS activity is too low *in vivo*, suggesting that aminoacylation of tRNA^{Arg} might easily become a limiting step for the translation machinery.

Mutations of positions 16 and 38 exhibited no lethal phenotype whatever the mutation was. No difference was

visible on the RNA blot profiles when compared with native tRNA^{Arg4}. Here again, the results show that the contacts observed in the structure of the ArgRS:tRNA^{Arg2} complex do not play a preponderant role, at least for the aminoacylation of tRNA^{Arg4}. Additionally, the tRNA^{Arg4}_{CCG} isoacceptor is the only arginine isoacceptor with U38 instead of A38. Thus, one can imagine that tRNA^{Arg4}_{CCG} uses a different binding mode of its anticodon loop, with a different distribution of the synthetase:tRNA interactions. It could therefore be interesting to crystallize the ArgRS:tRNA^{Arg4} complex, to see whether the binding mode is conserved.

Lethal mutations in the D loop lead to drastically reduced tRNA levels

Fourteen lethal mutants were identified in the D loop, in contrast to only three in the anticodon loop. Positions 14, 15, 18, 19 and 21 did not tolerate any change, except a unique A21C substitution. Due to their location, a first explanation might be that these mutations affect the tertiary interactions that stabilize the folded structure. It is well known that a complex network of interactions contribute to the stability of the tertiary structure of tRNA. The residues involved in these interactions are very conserved, like U8:A14 (reverse Hoogsteen pairing), G18:ψ55 and G19:C56 (distorted Watson-Crick base pair), or partially conserved, like the Levitt *trans* base pair G15:C48 (A15:U48 in tRNA^{Arg4}). In tRNA^{Arg4}, most of these interactions gave lethal phenotypes when they were disrupted by any change. In addition, they were not detected by northern blot, suggesting that these tRNAs are not produced or are rapidly degraded due to an unstable structure. A second cause of lethality might be reduced transcription. Transcription of eukaryotic tRNA genes is established by the RNA polymerase III transcription machinery that binds to the internal conserved sequences A and B mapped to nucleotides 13–20 and 51–64 (29). These sequences closely coincide with two conserved sequence blocks present in all eukaryotic tRNA genes, responsible for tRNA folding. The A and B blocks are bound simultaneously by the various subunits of the TFIIC factor that recruit RNA polymerase III (30). Thus, lethal mutations in positions 14, 15, 18 and 19 might affect transcription, but also later steps, like the processing or the stability of the transcripts. Such effects, in addition to transcription rate, have been shown with mutations that disrupt the tertiary interactions (31).

Among the selected mutants, the G19C lethal mutant is remarkable for two reasons. It is expressed and aminoacylated (Fig. 3), suggesting that the mutation may affect the following steps, like interactions with the translation machinery for instance (elongation factor or the ribosome). The *in vitro* aminoacylation parameters of this mutant were only affected at the K_m level (increase of 14-fold), an effect that could be explained by some structure change induced by the mutation. However, this affinity decrease measured *in vitro* had no effect on the steady-state level of aminoacylation *in vivo*.

The universally conserved U33 can be substituted

U33 is a quasi-universally conserved residue found in the anticodon loop of tRNAs (32). The U-turn motif associated with this residue was the first structural motif characterized in tRNA anticodon loops (33). It is defined by the formation of a hydrogen bond between U33 N3-H and O-P of residue 36 and

by a stacking interaction of the aromatic cycle of U33 and the OR atom of residue 35, leading to a turn in the phosphodiester backbone following U33. Despite this conserved structural role, a U33C viable mutant was isolated in tRNA^{Arg4}, whereas U33A and U33G were lethal. All were aminoacylated as efficiently as native tRNA (Fig. 3), suggesting that altered interaction with the ribosome coding site might explain the lethal phenotype. Interestingly, the same mutants were analyzed in amber suppressor *su7* for their ability to interact with the translation apparatus (34). Substitution U33C caused only a 2-fold decrease in suppression efficiency, a relatively modest effect that is in agreement with the viable phenotype that was observed in tRNA^{Arg4}. This corresponds to a 20-fold increase in the K_d value for ribosomes (35). On the other hand, when purines were introduced at position 33 a more pronounced increase of one order of magnitude was observed (34). In agreement with these effects, we observed a lethal phenotype when both A33 and G33 mutants were tested in the tR4 complementation assay.

Recent studies based on phylogenetic, structural and molecular dynamics data established a correlation between the presence of the conserved 33:35 interaction and a non-Watson-Crick isosteric base pair at position 32:38 (36). This arrangement would allow the formation of a canonical hairpin structure required for recognition by the ribosome and for the cognate interaction with the mRNA codon. However, this structural motif would also favor unfolding during the interaction with aaRSs when the tRNA anticodon bases are specifically recognized by the enzyme. U33 is found associated with a conserved pyrimidine at position 32 (98% of the occurrences), with a conserved purine at position 37 (71% of the occurrences) and with a strong preference for a purine at position 38. Earlier it was noticed that the first (position 32) and last residues (position 38) of the seven-membered anticodon loop are involved in a bifurcated hydrogen bond contact and form a 'pseudo' base pair (33). Most of the base pairs that are found at these positions cannot form classical Watson-Crick pairs, with the exception of the U·A base pair (32). Even in this case a classical pairing is not used, as shown by the crystal structure of tRNA^{Cys} complexed with elongation factor EF-Tu, where the pair Ψ32·A38 does not form a Watson-Crick arrangement, but is isosteric to Ψ·C and C·A observed in yeast tRNA^{Asp} and yeast tRNA^{Phe}. Among the six mutants that we constructed at positions 32 and 38 (U32·A38, U32·C38, U32·G38, A32·U38, C32·U38 and G32·U38) we found two Watson-Crick pairs and four non-Watson-Crick pairs. Some of them are rarely found, like A32·U38 (0.4%), C32·U38 (1.3%) and G32·U38 (0.6%) (36). However, all these mutants were functional and able to complement the tRNA^{Arg4} knockout strain, suggesting that they can pair with isosteric properties compatible with the formation of a canonical hairpin structure. In fact U·A, U·C and G·U pairs should be isosteric to C·A, as observed in the crystal structure of yeast tRNA^{Phe}, whereas U·G, C·U and A·U pairs should be isosteric to a U·U pair, as observed in the crystal structure of the tRNA^{Gln} complexed with GlnRS (36,37).

Another crucial residue for the anticodon loop of tRNA^{Arg4}_{CCG} is residue 37, which is methylated at position 1 (38). A G37U mutant is lethal in the knockout strain despite expression and aminoacylation levels comparable to native tRNA^{Arg4}. This strongly suggests a defect at the translation

level. Residue 37 is almost always modified and it has been proposed that this modification is essential for reading frame maintenance (39,40) and binding efficiency with the ribosome (41). Only very few tRNAs have a U at position 37, and among them most are mitochondrial tRNAs (32), thus it was not completely unexpected to find this inactive phenotype.

Concluding remarks

We have used a genetic screen to select from among mutated tRNA genes those that were transcribed into molecules inactive in translation of CGG arginine codons. A first class of inactive tRNAs exhibited transcription or stability deficiencies as shown by their absence from RNA samples. The second class of inactive tRNAs probably resulted from a decrease in their interaction capacity with the translation machinery. A third class of mutations that removed sequence-specific interactions between tRNA residues 16, 20 and 38 and ArgRS did not lead to lethal effects. Only moderate effects on the steady-state level of aminoacylation of U20 mutants were observed. This suggests that interactions with the anticodon triplet play the major role in arginine identity, as demonstrated by *in vitro* studies (14). Further *in vivo* investigations will focus on the anticodon triplet of tRNA^{Arg4} in order to explore the effects of the mutations on the arginine identity. The main advantage of the cellular assay is that it can take into account many cellular parameters that might contribute to amino acid identity. Among them, competition with other aaRS, the enzyme/substrate ratio and interaction with other cellular compounds or structures are parameters that differ with the *in vitro* assay conditions and that could significantly influence the aminoacylation of mutants (42,43).

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