tRNA nucleotide 47: An evolutionary enigma

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ABSTRACT

A previous analysis of tRNA sequences suggested a correlation between the absence of a nucleotide at position 47 (nt 47) in the extra loop and the presence of a U13:G22 base pair in the D-stem. We have evaluated the significance of this correlation by determining the *in vivo* activity of tRNAs containing either a C13:G22 or a U13:G22 pair in tRNA molecules with or without nt 47. Although this correlation might reflect some malfunction of tRNAs lacking nt 47, but containing the C13:G22, assays of the *in vivo* suppressor activity showed that this tRNA is actually more active than the tRNA with the features found in the database, i.e., a U13:G22 base pair and no nt 47. Moreover, analogous constructs with a GGC anticodon permitted the growth of an *Escherichia coli* strain deleted for tRNA^{Ala}_{GGC} genes equally well. On the other hand, long-term growth experiments with competing *E. coli* strains harboring the tRNA lacking nt 47, either with the C13:G22 or the U13:G22 base pair demonstrated that the U13:G22 tRNA overtook the C13:G22 strain even when the starting proportion of strains favored the C13:G22 strain. Thus, the preference for the U13:G22 tRNA lacking nt 47 in the sequence database is most likely due to factors that come into play during extended growth or latency rather than to the ability of the tRNA to engage in protein synthesis.

Keywords: amber suppression; competitive growth; evolution; structure; tRNA

INTRODUCTION

Sequence and structural databases are now sufficiently extensive to permit detailed inferences linking structure and function (Steinberg et al., 1993). This situation is fortunate because, in the case of tRNAs, where the art of sequencing has been practiced for more than 35 years, many aspects of the structure/function correspondence remain obscure, in spite of the availability of the three-dimensional structure (Dirheimer et al., 1995). For example, structural correlations between the D-stem and extra loop of tRNA were noted (Dirheimer et al., 1995). Steinberg and loudovitch (1996) recently have made a novel observation: the vast majority of cytoplasmic tRNAs that lack nt 47 contain a wobble U13:G22 base pair, even though this base pair is overwhelmingly populated by C13:G22 in tRNAs with nt 47. Notably, all exceptions to this correlation are tRNA^{Gly}, some of which are known *not* to promote ribosome-bound protein synthesis. The structural rationale of the correlation could be due to the fact that a normal Watson–Crick base pair at 13:22 cannot be accommodated without a disruption of the tertiary interaction

between the D stem and the shortened variable loop (Steinberg & loudovitch, 1996). Curiously, analysis of mitochondrial tRNAs revealed that almost half of the tRNAs examined contain the G:C base pair at 13:22 and lack nt 47.

In order to establish a possible functional basis for the correlation observed with cytoplasmic tRNAs, we have constructed tRNA genes containing either the wobble U13:G22 or the Watson–Crick C13:G22 base pair, both in the absence of nt 47, to test their *in vivo* activity. We show here that tRNAs lacking nt 47 with the C13:G22 base pair are actually more active in suppression of amber codons, whereas the two tRNAs have comparable activities in more normal protein synthesis. However, cells harboring tRNAs lacking nt 47 with the U13:G22 base pair outgrow cells with the C13:G22 tRNA in experiments of long-term growth incorporating significant periods of latency.

RESULTS

The presence of a U13:G22 wobble base pair in the vast majority of tRNAs lacking nt 47 suggests that some “selective” pressure must be exerted on this base pair and nt 47 in order to maintain it in archaeal, eukaryotic, prokaryotic, and chloroplastic tRNAs. It might follow

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from this conjecture that these U13:G22 tRNAs possess a greater biological activity than their C13:G22 Watson–Crick base pair counterparts. We have thus set out to evaluate and compare the *in vivo* activity of tRNAs constructed with these features.

Amber suppression assays

Suppression of amber mutations in *Escherichia coli* was first attempted to evaluate the *in vivo* activity of tRNA gene constructs incorporating the appropriate features. These constructs derived from the *E. coli*

tRNA^{Ala}_{UGC} gene sequence (Gabriel et al., 1996) were a wild-type tRNA^{Ala} suppressor (WT_{am}) control that contained the CUA anticodon to confer amber suppression ability (Fig. 1A) and two variants of WT_{am} lacking nt 47. The variants contained, in one case, a C at position 13 (C13_{am}), providing a C13:G22 base pair as in WT_{am}, and the other, a U at position 13 (U13_{am}), providing a U13:G22 wobble pair. These potential suppressor genes, WT_{am}, C13_{am}, and U13_{am}, were amplified by PCR from synthetic oligonucleotides and cloned into the pGFIB vector as described in Materials and Methods. The plasmids containing

FIGURE 1. Amber suppression assays. **A:** Sequence of the three suppressor tRNAs used in this study, nt 13, 22, and 47, are shown in bold and the amber anticodon CUA is underlined. The WT_{amb} suppressor has the same sequence as the *E. coli* tRNA^{Ala}_{UGC} except for the anticodon, whereas U13_{amb} and C13_{amb} lack nt 47 and have a U or a C in position 13, respectively. Oligonucleotides corresponding to these sequences were amplified by PCR and cloned in pGFIB. **B:** β -Galactosidase assays using the *E. coli* XAC-1 strain harboring the pGFIB plasmid constructs containing the suppressor tRNAs. Values shown are the mean of four independent experiments and have been normalized to the suppression activity of the WT_{amb} determined at 37°C. Similar results were obtained at 25 and 42°C (not shown). **C:** Growth of cells harboring one of the suppressors in Figure 1A or the plasmid pGFIB alone in minimal medium A (lacking arginine). Cells were diluted 200-fold from an overnight LB culture and grown at 37°C. Similar results were obtained at 30°C (not shown).

the genes and the plasmid alone were used to transform the XAC-1 strain of *E. coli*. This strain contains two reporter genes: a *lacI-lacZ* fusion and the *ArgE* gene, both of which possess an amber codon near the 5' end of their reading frame (Normanly et al., 1986).

After transformation, the level of suppression of each transformant was assayed using the standard β -galactosidase activity assay (Miller, 1972). Contrary to the expectation that the C13_{am} construct might be impaired, Figure 1B shows that C13_{am} is more active than U13_{am}: the activity order is WT_{am} ~ C13_{am} > U13_{am}. To extend these data, these experiments were repeated using the tRNA gene insertions into the medium-copy plasmid pMPM-A5 Ω (Mayer, 1995). Suppression assays in these experiments fully supported the order of activities established above. Moreover,

all constructs performed qualitatively as well in growth in the absence of arginine (Fig. 1C).

Growth assay in an *E. coli* tRNA^{Ala} deletion strain

Because suppression is a rather specialized form of translation, we wished to assess the activity of these tRNAs in more normal protein biosynthesis as well. The strain K45s1 Δ *ArelA* (Gabriel et al., 1996) contains deletions of the two tRNA^{Ala}_{GGC} genes and one of the three tRNA^{Ala}_{UGC} genes, and thus requires a vector-encoded tRNA^{Ala} gene with the anticodon GGC for normal growth. tRNA constructs for this assay were made by incorporating the anticodon loop from tRNA^{Ala}_{GGC} (Fig. 2A) into the wild-type sequence of tRNA^{Ala}_{UGC}. The two variants of this parent tRNA

FIGURE 2. Growth assay in an *E. coli* tRNA^{Ala}_{GGC} deletion strain. **A:** Sequence of the three tRNA_{GGC} used in this study, nt 13, 22, and 47, are shown in bold and the anticodon GGC is underlined. WT_{GGC} has the same sequence as the *E. coli* tRNA^{Ala}_{UGC} except that the anticodon loop (positions 32–38) is that of tRNA^{Ala}_{GGC}. U13_{GGC} and C13_{GGC} lack nt 47 and have a U or a C in position 13, respectively. Oligonucleotides corresponding to these sequences were amplified by PCR and cloned in pGFIB. **B:** Growth of *E. coli* K45s1 Δ *ArelA* cells harboring one of the tRNAs of Figure 2A in LB medium at 30°C. **C:** Growth of *E. coli* K45s1 Δ *ArelA* cells harboring one of the tRNAs of Figure 2A in LB medium at 37°C.

(WT_{GCC}) lacking nt 47 were designed with either a C13 (C13_{GCC}) or a U13 (U13_{GCC}, see Fig. 2A). As above, these genes were amplified by PCR and cloned in pGFIB. After transformation, growth curves of cells containing the different plasmids were obtained under various culture conditions and then compared. Among the variety of conditions that were used are different temperatures (30, 33, and 37 °C), liquid growth, and growth on split agar plates (see Materials and Methods). Under these conditions, cells harboring the different tRNA constructs exhibited essentially the same growth rate within the error margins of the experiments. Figure 2B and C illustrate the growth curves at 30 and 37 °C.

This system was further characterized by the determination of the *in vivo* level of tRNA aminoacylation by acid gel electrophoresis. As shown in Figure 3A, the levels of aminoacylation for these tRNAs are virtually indistinguishable; however, both tRNAs lacking nt 47 exhibit similarly lower steady-state levels than WT_{GCC}. Although lower levels could mirror either lower expression or faster degradation, the absence of higher molecular weight bands might rule out explanations based on defective tRNA maturation.

A potentially significant observation is that the C13_{GCC} construct has a distinctly faster gel migration than the other tRNAs (Fig. 3A, lane 5). Similarly, faster migration is also seen for the aminoacylated C13_{GCC} species (lane 6), suggesting that unusual 3' truncations would not be at the root of these observations. Moreover, primer extension experiments with this tRNA and a primer hybridizing to the 3' terminus of the molecule confirmed that a 5' processing difference was an unlikely explanation. On the other hand, our data do not address explanations involving differences in formal charges or conformations among the tRNAs.

Inspection of the tRNA^{Ala}_{UGC} sequence underlined the presence of a 7-methylguanosine (m⁷G) residue at position 46 (Steinberg et al., 1993). The frequent presence of m⁷G at this adjoining residue and its formal charge raised the attractive possibility that the absence of this modification might explain the aberrantly rapid migration of C13_{GCC}. We addressed this possibility biochemically by employing the sodium borohydride/aniline cleavage reaction of m⁷G (Zueva et al., 1985) combined with the primer extension technique to amplify any cleavage. If m⁷G46 were present, then it would be cleaved in borohydride/aniline, stopping reverse transcription at this position. However, these experiments did not reveal any clear difference between the RNA extracted from cells harboring C13_{GCC} and U13_{GCC} (Fig. 3B). We note that the total RNA preparation used in all of these experiments contains the endogenous wild-type tRNA^{Ala}_{GCC}, which does have the m⁷G46 and the probe we used would also recognize this tRNA. Therefore, our inability to see any differences between the extracts with C13_{GCC} and U13_{GCC} must be taken in light of a background consisting of a cleavage product

FIGURE 3. Characterization of tRNAs. **A:** tRNA aminoacylation assay. Total RNA was extracted from K45s1Δ*ArelA* cells harboring the tRNAs in Figure 2A, and subjected to PAGE under acid conditions (Bourdeau et al., 1998). The resulting gel was analyzed by northern blot hybridization with oligonucleotide probes complementary to the tRNA anticodon stem-loop and positions 34–53 of *E. coli* 5S RNA, as a control for the total amount of RNA. Prior to electrophoresis, an aliquot of the RNA was deaminoacylated in base (lanes +). Positions of the bands corresponding to the charged and uncharged tRNAs are indicated and the percentage of the aminoacylated form is noted below the lane. **B:** Summary of the results of the cleavage of tRNAs with sodium borohydride/aniline. Total RNA was deaminoacylated as in Figure 3A and then treated or not with sodium borohydride and aniline. The resulting uncharged RNA was reverse transcribed from a primer hybridizing to the 3' terminus of the tRNA^{Ala}_{GCC} and the mixture was then analyzed by PAGE and autoradiography. Two main bands corresponding to the tRNA cut at position 46 or not were obtained. These bands were quantified and the proportion of cleaved tRNA was calculated. These results are the compilation of three independent experiments.

of the endogenous tRNA, which would migrate slightly slower than the expected product for these variant tRNAs.

In addition, we tested our hypothesis genetically by expression of the tRNA genes in the GM18 strain of *E. coli*, which harbors a mutation in the *trmB* gene, encoding a guanosine tRNA methyltransferase responsible for m⁷G (Marinus et al., 1975). However, the mobility of the tRNAs was the same as that in the K45s1Δ*ArelA*

strain (data not shown), indicating that either a defect in this process is not the cause of the aberrant migration of C13 or the product of *trmB* is not responsible for tRNA^{Ala} G46 methylation in *E. coli*.

Long-term growth

As suggested previously, the basis of the fixation of a gene in a population is perceived to be based on its biological activity. However, under natural conditions, other factors might play roles of importance, for example, survival during latency or stability over long periods of active growth. Nevertheless, none of the *in vivo* experiments performed until now have evaluated factors other than short-term activity. To redress this situation, we have devised experiments dealing with long-term competition between the C13- and U13-containing tRNAs in a strategy similar to that used to study the effect of nonlethal mutations in yeast (Li et al., 1996). XAC-1 cells that had been transformed with either the C13_{am} or U13_{am} tRNA-containing plasmids were grown overnight to equivalent cell densities as determined by plating dilutions. The overnight cultures were diluted together into minimal medium lacking arginine so that selective pressure was exerted on the cells based on their ability to suppress the amber mutation of the *ArgE* gene. To assure ourselves that the outcome of the competition was not simply the consequence of a small, initial preponderance of the U13_{am} species, the amount of the C13_{am} inoculate used was 1.5 times that of the U13_{am}. The culture mixture was grown to saturation by incubating overnight at 30°C, and serial dilutions into fresh sterile medium were performed at every 24-h interval for up to three weeks thereafter. Analytical samples were also taken at regular intervals: plasmid DNA was isolated, digested with *Pvu* II, Southern blotted, and hybridized with a probe complementary to either the anticodon stem/loop or the acceptor/T stem region. Because *Pvu* II cuts the pGFIB on both sides of the multicloning site, as well as in the D-stem of the C13_{am} variant only, analysis of the ratio between the two variants in the culture could be accomplished by simple comparison of the gel band intensities: bands of 197 bp coming from the C13_{am} variant and 333 bp coming from the U13_{am} variant (Fig. 4A).

After 21 days (approximately 200 generations), 17 of 24 independent cultures exhibited proportions of the U13_{am} above 70% (Fig. 4B). Four other cultures were composed of 50–70% of the U13_{am}-bearing strain, whereas only three cultures still had a preponderance of the C13_{am} species (32%, 18%, 16% of U13). In fact, there seems to be more fluctuation between the various cultures at day 21 than five days before, and about half of the cultures have decreasing levels of U13 during these final days. As shown in the summary of these data (Fig. 4C), the proportion of the U13_{am}-bearing strain

in the culture gains significance above the initial ratio in about 16 days (*t*-test, $p \ll 0.001$).

A different experiment was performed in a chemostat, which allows continuous log-phase growth due to the constant addition of fresh medium. A 300-mL culture inoculated with U13_{am} and C13_{am} strains as above was grown without interruption for two weeks at an OD₆₀₀ between 1.4 and 1.6. This period should produce the same number of generations as the serial dilution experiments of above. Aliquots were collected daily to isolate and analyze DNA by hybridization as above. However, in contrast to the serial dilution experiment, Figure 4D demonstrates that the ratio of the two strains remains constant during the growth period.

DISCUSSION

The observation that all cytosolic tRNAs lacking a nucleotide at position 47 contain a U13:G22 base pair (except for some tRNA^{Gly}) prompted a series of experiments to place this correlation into a structure/function context. The results presented here imply that this correlation is not due to the “activity” of the tRNA in protein synthesis, as might have been expected. To the contrary, we show that a suppressor tRNA lacking nt 47 with the C13:G22 base pair is actually more active than the U13:G22 species in suppressing amber stop codons in the *lacZ* and *argE* genes (Fig. 1). Because suppression is a rather special case of protein synthesis, we deemed it necessary to extend our study to mimic more faithfully tRNA function in elongation with the aid of *E. coli* strain that required a plasmid-borne tRNA to decode the tRNA^{Ala}_{GCC} codons. These experiments confirmed the previous results on suppression in that tRNAs without nt 47 and the C13:G22 base pair are at least as active as those with the U13:G22 base pair (Fig. 2). Therefore, the correlation between the 13:22 base pair and nt 47 observed in the database must result from factors other than reliance on simple protein-synthesis activity. These reflections led to consideration of phenomena that may require more than several generations to express themselves, and we searched for a protocol that might test more realistically the constraints of life in a more natural environment (Li et al., 1996). Support for our tenet was obtained when we showed that competition between the strain harboring C13:G22 and U13:G22 produces a situation where the latter has a growth advantage after many generations (Fig. 4). In contrast, chemostat experiments showed that the phenomenon behind the selective advantage of the U13:G22 species could not be due solely to the number of generations, but possibly to a process that is active in denser, slower, or even the latent phase of bacterial growth.

To complement these studies, biochemical characterizations of the tRNA variants were performed. First, we showed that the level of aminoacylation does not

FIGURE 4. Long-term growth. **A:** Example of competition between cells with U13_{am} and C13_{am}. After inoculation, plasmid DNA was extracted from cells at different intervals, digested with *Pvu* II, and subjected to agarose gel electrophoresis and Southern blot hybridization with an oligonucleotide probe complementary to the tRNA anticodon stem-loop. The first two lanes show the results from cells with only the U13_{am} or C13_{am} species. **B:** Superimposition of the proportion of U13_{am} contained in the 24 independent cultures as a function of time. **C:** Summary of the data presented in Figure 4B. **D:** Continuous chemostat culture. XAC-1 cells with U13_{am} and C13_{am} were co-cultivated in minimal medium (lacking arginine) for 14 days at 30 °C (log-phase growth). Aliquots were collected daily, and DNA was purified and treated as above.

differ between the U13:G22 and C13:G22 tRNA species (Fig. 3A). Misaminoacylation is an unlikely cause for the observed correlation, because the correlation between the lack of nt 47 and the base pair at 13:22 is valid for all species of tRNAs even though each tRNA family has very different aminoacylation determinants (Martinis & Schimmel, 1995; McClain, 1995; Pallanck et al., 1995). Posttranscriptional processing of the tRNA was also considered following the observation of an aberrant gel migration of the tRNA lacking nt 47 with a C13:G22 base pair; however, modified nucleotide and primer extension analyses rule out maturation as a cause. Several attempts were also made to identify a

possible difference in the level of the m⁷G-modified nucleotide, often found at position 46 in tRNAs^{Ala}. This modification has a net positive charge, so its absence could account for the mobility shift of the C13:G22 species. However, an *E. coli* strain lacking the methyl transferase responsible for m⁷G46 formation exhibits no abnormal phenotype (Marinus et al., 1975) and our results suggest clearly that methylation of G46 could not be at the root of this observation. Neither do we have any direct evidence that the aberrant gel migration could be due to an unusual conformation of the tRNA, although an intriguing hypothesis might be that aberrant tRNAs lacking some tertiary interactions as predicted

to be at the base of this correlation (Steinberg & loudovitch, 1996) are less stable and thereby prone to more rapid degradation.

The reason why mitochondrial tRNA sequences do not follow this correlation so tightly observed for cytosolic tRNAs (Steinberg & loudovitch, 1996) might have remained mysterious if the tRNAs containing a Watson–Crick 13–22 base pair and no nt 47 (and thus no tertiary interaction 22–46) were inactive. However, the exceptional mitochondrial tRNA distribution can now be explained much more easily, because we show that the defect in these unusual tRNAs does not involve activity. Only a slight lowering of any threshold for efficiency, accuracy, or stability in mitochondrial tRNA function would be sufficient to allow retention of these unusual structures. This model implies that tRNA sequence variation generated during evolutionary drift occurs both in the cytoplasm and the mitochondria, but tighter selection occurs in the cytoplasm.

A more careful inspection of the sequence database can provide clues as to how mitochondria came to have tRNAs lacking the correlation between nt 13 and 47. First, these data show that there is almost never a compensation in nt 13 when nt 47 is missing in mitochondrial tRNA, supporting our assertion that mitochondria have higher tolerance. The data presented (Steinberg & loudovitch, 1996) also make clear that the absence of nt 47 in itself is undesirable: only 15% of cytosolic tRNAs lack this nucleotide. Our results seem to point in this direction as well; tRNAs containing nt 47 always had superior properties in terms of the level of suppression and protein synthesis than did the tRNAs lacking nt 47. The reason that most of mitochondrial tRNAs lack this nucleotide in spite of the inferences made from the cytosolic tRNAs may be due to forces acting to reduce the size of the mitochondrial genome. This hypothesis is supported by the fact that most of the abbreviating features (shortened rRNAs, tRNAs lacking entire domains, non-universal genetic codes, and relaxed rules of anticodon–codon interaction) are found in animal mitochondria (Benne & Sloof, 1986; Osawa et al., 1992) where, interestingly, more than 90% of the tRNAs lack this nucleotide (Steinberg et al., 1993; N. Cermakian, unpubl. obs.).

Lang et al. (1997) have sequenced and analyzed the mitochondrial genome of the early-diverging protist *Reclinomonas americana*. They showed that this genome has successfully resisted the genome-reducing force in mitochondrial genomes and thus retains many more bacterial features than other mitochondrial genomes. We inspected the *R. americana* mitochondrial tRNA sequences and found that only one does not satisfy the rule for the 13:22 base pair and the lack of nt 47, and this exception is a glycine tRNA, as are the other nine cytoplasmic exceptions observed previously. Also, only two of the complement of 19 mitochondrial tRNAs of *R. americana* lack nt

47, a proportion close to that of cytosolic tRNAs (15%), and far below that of mitochondrial tRNAs (65%) from other organisms.

However, the force of reduction of the mitochondrial genome cannot by itself completely explain the high rate of sequence and structure variation in tRNAs of more derived mitochondria. Atypical tRNAs are found in larger mitochondrial genomes, namely in mitochondria of plants, fungi, and protists, and, in animals, the rate of nucleotide substitution in tRNAs is 5–25 times greater in mitochondria than in the nucleus (Lynch, 1996). Kumazawa and Nishida (1993) have presented various possible factors for this rate difference: (1) higher mutation rate of mitochondrial DNA, possibly due to insufficiency of damage-repairing mechanisms; (2) absence of promoter elements within tRNA genes, in contrast with nuclear RNA polymerase III promoters; (3) absence of some processing sequence signals; (4) simpler and hence less constrained interaction with aminoacyl-tRNA synthetases and with translation factors. To these can be added the fact that tRNAs fulfill other roles in cytoplasm, but apparently do not in mitochondria (Söll, 1993).

Sequence correlations such as the subject of this study are the fruit of natural selection acting upon a population of cells containing different tRNA sequence variants. During the selection process, it is assumed that those cells harboring an active gene would be propagated, whereas one harboring a disabled or inactive gene would be eliminated. However, selection is a deceptively simple theoretical concept and biological process; real issues in selection may still be more complicated than some of our results might lead one to believe.

MATERIALS AND METHODS

Plasmid construction

The strain GM18 was obtained from the *E. coli* Genetic Stock Center (New Haven, Connecticut; CGSC #5384). All suppressor and tRNA genes were prepared by PCR amplification of oligodeoxynucleotide templates corresponding to the sequences in Figures 1A and 2A and flanked by *EcoR* I and *Pst* I restriction sites. A mixture containing 100 ng of this template, 100 pmol of each primer (designed from positions 1–12 and 63–76 of the tRNA), 200 μ M of dNTPs, and 2 U of Vent DNA polymerase (NEB) was heated 4 min at 95 °C, followed by 30 cycles of: 30 s at 95 °C, 30 s at 72 °C; and then 4 min at 72 °C. The double-strand DNA products were digested with *EcoR* I and *Pst* I and cloned in the pGFIB1 plasmid (Masson & Miller, 1986), cut with the same enzymes, and treated with Calf Intestinal Alkaline Phosphatase. The identity of the inserts was confirmed by sequencing using the dideoxy method (Pharmacia). Transformations were performed by electroporation or heat shock. Freshly transformed cells were used in all experiments.

Characterization of tRNAs

The level of in vivo tRNA aminoacylation was obtained by analysis of total RNA extracted under acid conditions on an acid 6.5% polyacrylamide gel followed by transfer to a nylon membrane (Amersham) and hybridization with an oligonucleotide probe complementary to the anticodon stem-loop, as described in Varshney et al. (1991) and Bourdeau et al. (1998). Primer extension experiments were performed as in Sambrook et al. (1989), using a probe complementary to positions 54–76 of the tRNA^{Ala}_{UGC}. Fragmentation of RNA with sodium borohydride and aniline was as in Zueva et al. (1985), with modifications: 6–8 µg of total RNA (in 1 or 2 µL) was treated with 1 µL of 0.5 M Tris, pH 9, for 30 min at 37 °C to deaminoacylate tRNAs. Then, 9.5 µL of 1 M Tris-HCl, pH 8, and 8 µL of 1.25 M NaBH₄ were added, and the mixture was incubated for 5 min at 20 °C in the dark. After ethanol precipitation, the pellet was resuspended in aniline:acetic acid: water (1:3:7) and incubated 10 min at 60 °C. The RNA was precipitated, and either applied to a gel for analysis or subjected to primer extension.

Suppression and tRNA complementation assays

The XAC-1 (F' *lacI373lacZ_{m118am} proB+*/F[−] Δ(*lac-proB*)XIII *nalA rif argE_{am} ara*) strain of *E. coli* was used for suppression assays (Normanly et al., 1986). An overnight culture grown at 37 °C in LB medium containing ampicillin was diluted 1:50 into minimal medium A containing 0.4% glucose, 1 mM MgSO₄, 20 µg/mL vitamin B1, 50 µg/mL ampicillin, and 50 µg/mL L-arginine. After growth at 30 or 37 °C, β-galactosidase activity was determined using the method of Miller (1972). Suppression of the stop codon in the *ArgE* gene was assessed by monitoring the optical density at 600 nm of cells growing in a minimal medium A as above, but lacking arginine, following dilution of an overnight culture in LB medium containing ampicillin.

The growth properties of the tRNA^{Ala}_{GGC} deletion strain, K45s1Δ*AreIA* (*glyV55* Δ(*tonB trpAB17*) Δ(*ArgF lac*)):TN10-5, *relA1*, Δ*rrnA*::*camR*, Δ*ala2*::*kanR*/F' *trpA*(UGA15)) expressing the wild-type and mutant tRNA^{Ala}_{GGC} constructions were evaluated by monitoring OD₆₀₀ of a culture in LB medium containing ampicillin at 30 or 37 °C, or as in Gabriel et al. (1996): 2 µL of an early-log phase (OD₆₀₀ = 0.3–0.5) culture were spread on split-agar plates containing LB medium on one side and minimal medium A (containing 0.2% glucose or glycerol, 1 mM MgSO₄, 5 µg/mL vitamin B1, 40 µg/mL L-arginine, 40 µg/mL L-tryptophane, 50 µg/mL ampicillin, and 0.012% bromothymol blue) on the other. These plates were then incubated at 30, 33, or 37 °C.

In vivo competition experiments

Growth competition experiments were performed in sterile 24-well plates (Falcon 3047), using XAC-1 cells transformed with either the U13_{am} or C13_{am} tRNA species. Twenty-four overnight LB cultures of each clone were diluted and mixed in 500 µL of minimal medium A containing 0.4% glucose, 1 mM MgSO₄, 20 µg/mL vitamin B1, 50 µg/mL ampicillin without arginine. Each preculture in LB was inoculated with a different colony. U13_{am} was diluted 0.8:1,000 and C13_{am} 1.2:1,000.

The viable cells in precultures were counted after plating dilutions on LB, to confirm that more cells containing C13_{am} than U13_{am} had been used in the inoculation. The cultures were incubated with agitation at 30 °C and an aliquot was diluted 1:500 into fresh medium every day. After 1, 6, 11, 16, and 21 days, cells were pelleted and plasmid DNA isolated and then digested with *Pvu* II. This restriction enzyme cuts at CAGCTG, which is found on either side of the multicloning site in pGFIB, and also in the *E. coli* tRNA^{Ala} gene (positions 13–18), but not in the U13_{am}. Digestions were analyzed on 1.5% agarose gels, Southern blotted to Hybond-N nylon membrane (Amersham), and hybridized with an oligonucleotide probe downstream of the *Pvu* II site in the tRNA gene. *Pvu* II cleavage yields a 197-bp digestion product in the case of C13_{am} and a 333-bp fragment for U13_{am}. Films were scanned and the relative amount of each species in each sample was evaluated using the ImagePC program (Scion Corporation, release Beta 1).

Continuous growth experiments were performed in a Bioflo bench top chemostat model C30 (New Brunswick Scientific Co.). In a pilot experiment without the addition of fresh medium, to determine the cell division time in logarithmic phase, a growth curve of the XAC-1 strain harboring C13_{am} or U13_{am} was performed at 30 °C in minimal medium A containing 0.4% glucose, 1 mM MgSO₄, 20 µg/mL vitamin B1, 50 µg/mL ampicillin without arginine. Under these conditions, doubling time was about 90 min. The XAC-1 strain harboring either the C13_{am} or U13_{am} plasmid were grown independently overnight in LB with ampicillin, and mixed in the culture vessel of the chemostat in 300 mL of minimal medium A (as in the competitions above). C13_{am} was diluted 1:1,200 and U13_{am}, 1:800. Incubation was performed at 30 °C for 7 h and from then media was added continuously. During the subsequent continuous culture, the temperature was kept at 30 °C and the addition of media was at the rate of 60–70 mL/h. Under these conditions, the OD₆₀₀ of the culture remained between 1.4 and 1.6, indicative of log phase. Aliquots were taken from the culture every day for two weeks, plasmid DNA was purified, digested, and analyzed by hybridization as above.

ACKNOWLEDGMENTS

We thank Alice Rae for critical reading of this manuscript, other members of this laboratory, particularly Sergey Steinberg, for helpful discussions, and Professor Patrick Hallenbeck (Université de Montréal) for the loan of the chemostat. We are equally grateful to Kay Gabriel and Jay Schneider (University of Wisconsin, Madison) for their technical help. R.C. is the Richard Ivey Fellow of the Canadian Institute for Advanced Research and N.C. is a FCAR (Québec) predoctoral scholar. This work has been supported by grants from the Natural Science and Engineering Research Council and the National Institutes of Health (GM42123).

Manuscript accepted without revision May 5, 1998

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