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Novel Transfer RNAs That Are Active in *Escherichia coli*†

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ABSTRACT: Many of the mammalian mitochondrial tRNAs contain significant nucleotide deletions in the dihydrouridine (D) stem or TΨC stem, so that they cannot fold into the canonical cloverleaf structure. This suggests that alternative forms and shapes are possible for a mitochondrial tRNA that functions in the specialized translational apparatus of the mammalian mitochondria. The question of whether significant structural alterations may be accommodated by a bacterial protein synthesis machinery, such as in *Escherichia coli*, is unanswered. In this work, all but ten positions in the gene for the 76-nucleotide coding sequence of an *E. coli* amber suppressor tRNA were permuted and screened for biological activity in vivo. Sequence analysis of a collection of biologically active variants established that many have unusual structures that include base-pair mismatches in helical stems, substitutions of normally conserved bases, and deletions. Independent mutations were obtained that weaken base pairs or tertiary interactions that normally stabilize the coaxial stacking of the D and anticodon stems, suggesting that the translational apparatus can accommodate considerable flexibility in this part of the molecule. The results demonstrate the capacity of the bacterial protein synthetic apparatus to accommodate altered tRNA structures that are not represented by any naturally occurring tRNAs.

Transfer RNAs (tRNAs) normally comprise 74–93 nucleotides that fold into a cloverleaf structure that is stabilized by internally self-complementary sequences. This cloverleaf consists of four double-helical stems and four single-stranded regions that are known as the dihydrouridine (D), anticodon, extra (or variable), and TΨC loops. The differences in the sizes of tRNAs are accounted for by variations in the D and extra loops. Within the cloverleaf, there is a set of 15 conserved and multiple semiconserved bases (either constant purines or pyrimidines) that establish a sophisticated network of tertiary interactions that stabilize an "L"-shaped structure (Kim et al., 1974; Robertus et al., 1974; Rich & RajBhandary, 1976). In this structure, the interactions between the invariant U8 and A14 and between the purines at positions 9 and 23 enable a tight turn from the amino acid acceptor stem to the D stem, so that the acceptor stem can stack directly on top of the TΨC stem to form one arm of the L, while the D stem stacks on the anticodon stem to form the other arm of the L (Figure 1). The two arms are then joined by interactions between the constant nucleotides in the D and the TΨC loops (at G18-Ψ55, G19-C56) and between those in the D and the extra loops (at G15-C48) to place the 3′ CCA terminal sequence and the anticodon at the opposite ends of the L, separated by about 75 Å. This tertiary arrangement is believed to be common to most tRNAs (Rich & RajBhandary, 1976).

The importance of selected tertiary interactions for the tRNA structure has been probed by the lead cleavage reaction (Behlen et al., 1990; Sampson et al., 1990), in addition to studies of the effects of these interactions on in vitro recognition by an aminoacyl-tRNA synthetase (Sampson et al., 1990). In this work, we addressed the requirement for a cloverleaf and an L-shaped structure for a tRNA during protein synthesis in vivo. We began with the G3·C70 variant of an *Escherichia coli* alanine amber tRNA (Figure 1 inset). The single substitution of a G3·C70 base pair for the wild-type G3·U70 in the acceptor stem eliminates the major identity determinant for this tRNA (Hou & Schimmel, 1988) so that G3·C70 $tRNA^{Ala/CUA}$ is a weak suppressor in *E. coli*. The use of a weak suppressor afforded the opportunity to select for mutants that, among other possibilities, resulted in a stronger suppressor phenotype.

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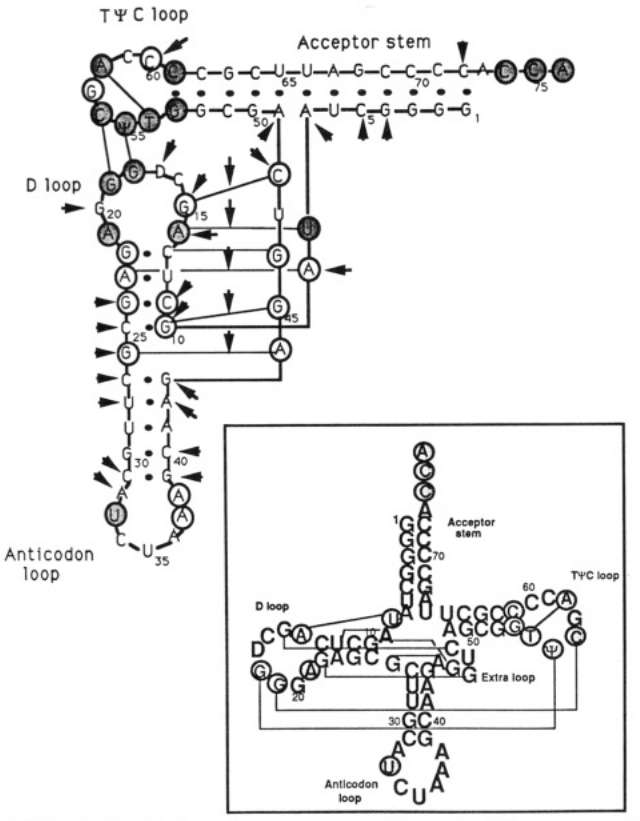

FIGURE 1: The L-shaped arrangement of the nucleotide sequence of G3·C70 tRNA$^{Ala/CUA}$, showing the stacking interactions that are found in yeast tRNA^{Phe}. The covalent connectivity of the 76 nucleotides in the polynucleotide chain is indicated by heavy lines. The 15 conserved nucleotides are indicated by shaded circles, whereas those of the semiconserved nucleotides are shown as open circles [see Rich and RajBhandary (1976)]. Secondary structure base pairings are shown by dots, while tertiary hydrogen-bonding interactions are shown by thin lines. Arrows indicate altered tertiary interactions and nucleotide changes that occurred among the 15 functional tRNA mutants analyzed in this study. Details of the changes are shown in Table I. Inset: sequence and cloverleaf structure of *E. coli* tRNA$^{Ala/CUA}$. Nucleotides at positions 33–38, 70, and 74–76 were maintained constant during the synthesis of the tRNA gene.

Materials and Methods

The gene that encoded G3·C70 tRNA$^{Ala/CUA}$ was synthesized in two degenerate DNA oligonucleotides (Applied Biosystems 380B DNA synthesizer): the first coded for nucleotides from positions 1 to 38 and an *Eco*RI restriction site at the 5′ end; the second coded for nucleotides from positions 33 to 76 and a *Pst*I restriction site at the 5′ end. The two oligonucleotides were of opposite polarity, so that they could be annealed at the overlapping sequence from positions 33 to 38. Permutation of bases was included at each step of the synthesis (except for nucleotides in the anticodon loop from positions 32 to 38, and nucleotides at the 3′ end from positions 74 to 76) at a frequency of 10%, i.e., 90% of the wild-type nucleotide and 3.3% each of the other three nucleotides.

The synthesized oligonucleotides were purified by a 20% polyacrylamide–7 M urea gel electrophoresis and were then phosphorylated by T4 polynucleotide kinase. Two hundred picomoles of each phosphorylated oligonucleotide, in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT (dithiothreitol), and 50 μg/mL BSA, was mixed, heated to 70 °C for 5 min, and allowed to cool to room temperature over a period of 60 min. After annealing, 0.5 mM dNTP and 5 units of Klenow DNA polymerase were added, and the reaction was incubated for 12 h at room temperature to allow the synthesis of the complementary strands using the single-stranded regions in the two oligonucleotides as templates. After synthesis, 10 pmol of the double-stranded tRNA gene, digested with *Eco*RI and *Pst*I restriction enzymes, was ligated into the corresponding sites in plasmid pGFIB-I. The ligation mixture was introduced into *E. coli* strain XAC-I (Kleina et al., 1990) (F′ lacI lacZ_{am} proB$^+$/F$^-$ Δ(lacproB) *nalA*, *rif*, *Arg*E_{am}, *ara*). Functional tRNA mutants were distinguished from the nonfunctional mutants by their ability to suppress the lacZ_{am} mutation of XAC-I, thereby appearing blue on LB plates that contained the X-Gal indicator substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranose.

Plasmid pGFIB-I (Normanly et al., 1986a,b) contains the strong *E. coli lpp* promoter 5′ to the *Eco*RI restriction site and a rrnC terminator sequence 3′ to the *Pst*I restriction site. Synthetic tRNA genes can be introduced between the *Eco*RI and the *Pst*I restriction sites, and the tRNA gene products can be functionally expressed in *E. coli* (Normanly et al., 1986). There is an F1 intergenic region in pGFIB-I so that single-stranded DNA of the plasmid can be prepared after addition of the helper phage M13KO7 for the purpose of sequence analysis. All sequence analyses of the mutants were performed by the dideoxy termination method (Sanger et al., 1974).

Results

In our mutagenesis scheme, 66 of the 76 nucleotides were subjected to mutagenesis at a 10% frequency. The probability of recreating a native structure that contained an average of 6.6 nucleotide substitutions (10% × 66 nt = 6.6 nt) is small (between 10^{-5} and 10^{-4}), because substitutions at many positions require compensatory mutations to preserve the structure. From the total tRNA mutants that were generated, a subset was introduced into *E. coli* strain XAC-I (Kleina et al., 1990) to test for their functional ability to suppress an amber mutation. Of approximately 10^5 tRNAs that were tested, the vast majority (>99%) were nonfunctional. Of the remaining 250 that were functional, sequence analysis of 82 suppressors established that 67 had the starting sequence and 15 contained sequence variations. Thus, about 82% of the 250 functional suppressors were of the starting sequence, which is of the order of what would be expected among a population of 10^5 transformants ($0.9^{66} \times 10^5 \cong 100$).

Each of the 15 functional variants had a unique sequence and was, therefore, independent. Each also conferred an Arg$^+$ phenotype to XAC-I, demonstrating that these suppressors were active in at least two different codon contexts. Their suppression efficiencies, determined by the amount of β-galactosidase produced by the *lac*Z_{am} gene (Kleina et al., 1990), were at least 20% of that of the starting G3·C70 tRNA$^{Ala/CUA}$.

The nucleotide substitutions of the 15 functional mutants are listed in Table I, and their locations are illustrated in Figure 1. Four mutants contain nucleotide substitutions that preserve the secondary and tertiary structure (AlaGC1–4), while the remaining 11 mutants contain in each at least one novel structural feature that includes nucleotide deletions, mismatches, and substitutions of the normally conserved or semiconserved bases. Although these features are also prevalent in nonfunctional mutants, the functional mutants in general have a smaller number of mismatches (1 vs 5) and harbor mutations at conserved and semiconserved nucleotides that stabilize a different set of tertiary interactions than those found in the nonfunctional mutants. Specifically, mutations at the tertiary interactions that stabilize the TΨC loop and the corner of the L are found only in the nonfunctional mutants, whereas mutations in nucleotides that are responsible for holding together the D–anticodon helix are found in the functional mutants.

Table I: Functional tRNA Mutants That Are Active in *E. coli*[a]

mutant	nucleotide substitutions	rel suppression efficiency	comments
AlaGC1	G20 → A20 A32 → G32 C60 → U60	0.59	three substitutions at positions that are free of structural constraints
AlaGC2	A49 → G49	0.86	introduce a G49·U65 pair in the TψC stem
AlaGC3	A7 → G7	0.27	introduce a G7·U66 pair in the acceptor stem
AlaGC4	C5 → U5	1.04	introduce a U5-G68 pair in the acceptor stem
AlaGC5	G4 → A4	0.77	introduce an A4·C69 mismatch in the acceptor stem
AlaGC6	G4 → C4	0.23	introduce a C4·C69 mismatch in the acceptor stem
AlaGC7	G20 → U20 C72 → A72	0.36	introduce a G1·A72 mismatch in the acceptor stem
AlaGC8	C27·G43 → U27·U43	0.50	creates a U27·U43 mismatch in the anticodon stem
AlaGC9	C31·G39 → A31·U39, ΔA49, C60 → A60	0.77	deletion of A49
AlaGC10	C5 → G5 A9 → U9 G20 → U20 C25 → U25 C26 → U26	0.27	introduce a G5·G68 mismatch in the acceptor stem, affect tertiary interaction at positions 9-12-23, and 26-44
AlaGC11	A9 → U9 G10 → C10 C11·G24 → G11·C24 A14 → C14 G26 → A26 U28 → A28 C40 → A40	0.36	affect tertiary interaction at positions 8-14, 9-12-23, and 26-44, introduce a C10·C25 mismatch in the D stem and A28·A42 and G30·A40 mismatches in the anticodon stem
AlaGC12	A9 → U9 D17 → A17	0.41	affect tertiary interaction at positions 9-12-23
AlaGC13	G15 → U15	0.23	affect tertiary interaction at positions 15-48
AlaGC14	G15 → U15 C48 → G48 A49 → G49	0.64	affect tertiary interaction at positions 15-48
AlaGC15	G26 → A26 A42 → C42 C60 → U60	0.96	affect tertiary interaction at base pair 26-44, introduce a U28·C42 mismatch in the anticodon stem

[a] Nucleotide substitutions in 15 functionally active mutants of G3·C70 $tRNA^{Ala/CUA}$ are given. All mutants suppressed the $lacZ_{am}$ and $ArgE_{am}$ amber alleles XAC-I. The relative suppression efficiency, determined by the β-galactosidase (Kleina et al., 1990), is given as a fraction of that of the starting G3·C70 $tRNA^{Ala/CUA}$. The suppression efficiency of the starting suppressor was 2.2%.

Three of the functional mutants have a wobble G·U base pair (AlaGC2–4), and seven others have mismatches in the helical stems (AlaGC5–8, -10–11, -15). The mismatches of AlaGC5–7 are all located in the acceptor stem while the mismatch in AlaGC8 is in the anticodon stem, where the D stem is joined. The latter may perturb the coaxial stacking between the D and the anticodon stems. While the helical stems of procaryotic and eucaryotic cytoplasmic tRNAs are primarily comprised of Watson–Crick base pairs, with usually no more than one G·U base pair per molecule, mitochondrial tRNAs have many mismatches in the stems, such as A·C and A·A, as well as G·U base pairs (Sprinzl et al., 1989).

Mutant AlaGC9 harbors a deletion of A49 in the TΨC stem. A49 normally pairs with U65 to form the first base pair of the TΨC stem upon which the A7·U66 base pair of the acceptor helix is stacked. The elimination of A49 could create a bulged U65 between the acceptor and TΨC stems. No natural tRNAs have an unpaired U65.

Three mutants (AlaGC10–12) contain a U9 that substitutes the wild-type A9 in a base triple, whereby A9 makes a Hoogsteen base pair with A23 (Kim et al., 1974; Robertus et al., 1974), which in turn is paired to U12 in the D stem. The U9-U12-A23 base combination of the three mutants is not found in existing tRNAs. AlaGC11 harbors an additional substitution at position 14 that changes the conserved A to C. The wild-type A14 pairs with U8 in a reversed Hoogsteen interaction (Kim et al., 1974; Robertus et al., 1974). Although C14 could in principle form two H-bonds with U8, it has never been found in existing tRNA sequences. Except for T5 phage $tRNA^{Asp}$, which has a G8·G14 combination, all cytoplasmic species have a U8·A14 pair. In mitochondria, while the base combinations at 8·14 are more variable, only bases A, G, and U are seen at position 14 (Sprinzl et al., 1989).

Mutants AlaGC13 and AlaGC14 contain a substitution of U15 for the wild-type G15 in the D loop. G15 forms a pair with C48 in the extra loop to continue to stack on U8·A14. Except for the *E. coli* and *Salmonella typhimurium* $tRNA_1^{Gly}$ (Hill et al., 1973), position 15 always has a purine and position 48 has a complementary pyrimidine (Levitt, 1969). However, an interchange of purine and pyrimidine, such as C15·G48 in yeast $tRNA^{Phe}$ (Sampson et al., 1990) and U15·A48 in yeast $tRNA^{Asp}$ (Perret, 1990; Giege et al., 1990), has been shown to have little effect on aminoacylation in vitro. The U15·C48 and the U15·G48 combinations described here are new and indicate that, in addition to aminoacylation, other steps in protein synthesis can accommodate alterations of the 15·48 tertiary interaction.

Mutants AlaGC11 and AlaGC15 substitute the wild-type G26, located at the junction of the coaxial stacked D–anticodon helix, with an A, while mutant AlaGC10 substitutes G26 with a U. These substitutions can affect the pairing of G26 with A44 in the extra loop, which normally stacks between the D and anticodon stems and maintains the continuity of stacking interactions therein. G26·A44 is the most common base pair in *E. coli*, although other base combinations such as A·U, A·C, C·G, and A·G occur frequently in various procaryotic and eucaryotic organisms. The G26 → U26 change can retain the same hydrogen-bonding capacity of the wild type, and the U26·A44 pair has been found in a yeast $tRNA^{Pro}$ and a human $tRNA^{Gly}$. Conversely, the G26 → A26 change

offers no hydrogen-bonding potential with A44, and yet the A26·A44 combination has also been observed in numerous mitochondrial tRNAs and in one isoacceptor of *E. coli* $tRNA^{Ser}$ and of yeast cytoplasmic $tRNA^{Val}$ (Sprinzl et al., 1989).

Discussion

The results summarized in Figure 1 indicate that the junction between the D stem and the anticodon stem may be quite flexible, because not only are secondary structure mismatches at 10·25 and 27·43 found here but there are substitutions of the conserved and semiconserved nucleotides that stabilize the tertiary stacking interactions at this junction (at 8-14, 9-12-23, and 26-44). It is worth noting that, while there are a total of seven substitutions in AlaGC11 distributed throughout the D–anticodon helix, the acceptor–TΨC helix is completely intact. The flexibility of the junction further raises the question of whether a portion of the D–anticodon helix might be dispensable for protein synthesis in *E. coli*, as is the case for mammalian mitochondria (deBruijin et al., 1980; Barrell et al., 1980; Bibb et al., 1981). It is well established that, at least for some tRNAs, the D–anticodon helix is not required for aminoacylation (Francklyn & Schimmel, 1989, 1990).

Except for mismatches, we have not found substitutions that can alter the tertiary interactions within the TΨC loop and between the TΨC and D loops. These tertiary interactions stabilize the corner of the L (Figure 1) and include the nucleotides referred to as the "conserved cluster" (T54·A58, G18·Ψ55, G19·C56, and G18-G19-G57) (Ladner et al., 1975). Substitutions of these nucleotides have been detected in many of the nonfunctional tRNA mutants, yet none is found in the 15 functional mutants that were analyzed. It is interesting to note that a nucleotide insertion between U60 in the TΨC loop and the conserved C61 in the TΨC stem enables a glycine tRNA to shift reading frame (O'Mahony et al., 1989). Thus, changing the TΨC loop structure may affect translational fidelity.

The novel nucleotide substitutions at positions 9 and 15 are of special interest. There are three independent mutants that harbor a change at position 9, and in all cases, the change is from A to U. Likewise, in the two independent mutants that alter the nucleotide at position 15, the change is from G to U. There may be selective pressure in favor of particular nucleotides at positions 9 and 15 such that they form new, alternative tertiary interactions. An example of alternative base pairing is found in a mammalian mitochondrial tRNA missing the entire D stem and loop. Here, the conserved U8 in the normal U8·A14 pair is postulated to pair with a lone nucleotide in the TΨC loop (deBruijn & Klug, 1983). This alternative base pairing, together with other compensatory interactions, allows a truncated cloverleaf structure to fold into a tertiary structure that is similar to that of yeast $tRNA^{Phe}$.

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