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LETTER TO THE EDITOR

A tRNA circularization assay: Evidence for the variation of the conformation of the CCA end

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The CCA end is common to all tRNAs as the universal site for amino acid attachment. It is also conserved in the 3′-terminal tRNA-like structure of viral genomes that can be aminoacylated by an aminoacyl-tRNA synthetase (Florentz & Giegé, 1995). During aminoacylation, the CCA end enters the catalytic center of an aminoacyl-tRNA synthetase and provides the site for chemistry to take place. The CCA end is also widely used in replication of retroviruses, the bacterial single-stranded RNA viruses, and duplex DNA plasmids of fungal mitochondria. During replication, the CCA end interacts with the template-specificity domain of reverse transcriptase or replicase and provides the initiation site for primer binding and extension (Maizels & Weiner, 1994). The importance of the CCA end in translation and in replication suggests that its conformation will play a role in these two fundamental processes.

Previous studies indicate that the N73 nucleotide and a C1:A72 mismatch have the ability to manipulate the conformation of the CCA end. An NMR analysis of tRNA acceptor stems (Puglisi et al., 1994) showed that the CCA end preceded by an A73 nucleotide has an extended conformation, whereas that preceded by a U73 has a fold-back structure. The fold-back structure of the CCA end brings the terminal A76 adjacent to the 5′ side of the acceptor stem and may be stabilized by a weak hydrogen bond between A76 and U73. The folded CCA end is also found in the crystal structure of the *Escherichia coli* initiator tRNA^{fMet} which, despite having an A73, carries an unusual C1:A72 mismatch that is thought to contribute to the fold-back of the CCA end (Schevitz et al., 1979; Woo et al., 1980; Ferguson & Yang, 1986; Pscheidt & Wells, 1986). In tRNA^{fMet}, C1:A72 is important for the formylation of methionine by the *E. coli* formylase (Guillon et al., 1992). Although substitution of C1:A72 with a Watson–Crick base pair generates a defect that eliminates the activity for formylation, this defect can be rescued by introducing U73 to replace A73 (Lee et al., 1992, 1993). The implication is that recognition of tRNA^{fMet} by the formylase is mediated through the fold-back structure of the CCA end such that either C1:A72 or U73 can confer substrate recognition.

We present here additional evidence that U73 and C1:A72 can influence the conformation of the CCA end. The evidence is based on the initial rate of tRNA circularization catalyzed by T4 RNA ligase. Previously, Bruce and Uhlenbeck (1978) used T4 RNA ligase to join the 5′ and 3′ ends of a tRNA and showed that the rate of circularization for *E. coli* tRNA^{fMet} is significantly faster than that of yeast tRNA^{Phe}, which has an extended CCA end (Kim et al., 1974; Robertus et al., 1974). One interpretation for the faster rate of circularization of tRNA^{fMet} is that the two ends of the tRNA are close to each other, which is consistent with the folded CCA end. Conversely, an interpretation for the slower rate of circularization of tRNA^{Phe} is that the two ends are further apart, which is consistent with the extended CCA end. Thus, the rate of tRNA circularization may be correlated with the conformation of the CCA end. Our goal is to examine this correlation in *E. coli* tRNA^{Cys}. Although the structure of *E. coli* tRNA^{Cys} is not yet available, we propose that this tRNA will recapitulate the structure of the NMR model substrate because it shares U73 and the first four base pairs in the acceptor stem (Puglisi et al., 1994; Walter & Turner, 1994). This raises the hypothesis that *E. coli* tRNA^{Cys} will have a folded CCA end and that the folded CCA end will facilitate circularization catalyzed by T4 RNA ligase. Our studies support various aspects of this hypothesis and provide evidence for the conformational variations of the CCA end among tRNAs.

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We show in Figure 1 that the initial rate of tRNA circularization is fast with the U73-containing wild-type (22.0 pmol/h), which is followed by the initial rate of the C73 variant (20.2 pmol/h). In contrast, the initial rate of circularization for the A73 and G73 variants is slow (2.0 pmol/h). This clearly establishes that a pyrimidine at N73 facilitates circularization, whereas a purine retards circularization. We also show that the preference for U73 in tRNA circularization can be extended to the acceptor stem of *E. coli* $tRNA^{Ala}$, which contains A73, and of yeast $tRNA^{Asp}$, which contains G73. Structural analyses of both indicate an extended CCA end (Ruff et al., 1991; Limmer et al., 1993). We show that the initial rate of circularization for both is poor and is similar to those of the A73 and G73 variants of *E. coli* $tRNA^{Cys}$ (2.0 pmol/h). However, introduction of U73 elevates the initial rate of *E. coli* $tRNA^{Ala}$ by 30-fold (to 60 pmol/h) and that of yeast $tRNA^{Asp}$ by 180-fold (to 360 pmol/h). These results demonstrate a general role of U73 to enhance circularization over A73 or G73.

Introduction of C1:A72 to *E. coli* $tRNA^{Cys}$ enhances the initial rate of circularization from 22 pmol/h of the wild-type to 37 pmol/h for the mutant. We show that this enhancement is not due to the introduction of C1, which is a better substrate than G1 for the T4 RNA ligase-catalyzed reaction (McLaughlin et al., 1985). Rather, this enhancement is due to the unpaired nature of C1:A72, because a C1:G72 variant does not enhance the initial rate of circularization of the wild-type. The initial rate of circularization of the C1:G72 variant (13.2 pmol/h) is actually slower than that of the wild type (22 pmol/h). The contribution of C1:A72 to the rate enhancement is even more pronounced in the context of the A73 variant. The intial rate of circularization of the C1:A72/A73 variant is 82.1 pmol/h, which is faster than that of the C1:A72/U73 variant (37.0 pmol/h).

Control experiments show that the product of our circularization assay is an intramolecular ligation but not an intermolecular ligation. An intermolecular would generate a linear tRNA dimer that can be topologically distinguished from a circular tRNA or a linear monomer by a site-specific cleavage. To prove the identity of the ligation product as a circular tRNA, we have purified the product and compared its cleavage pattern with

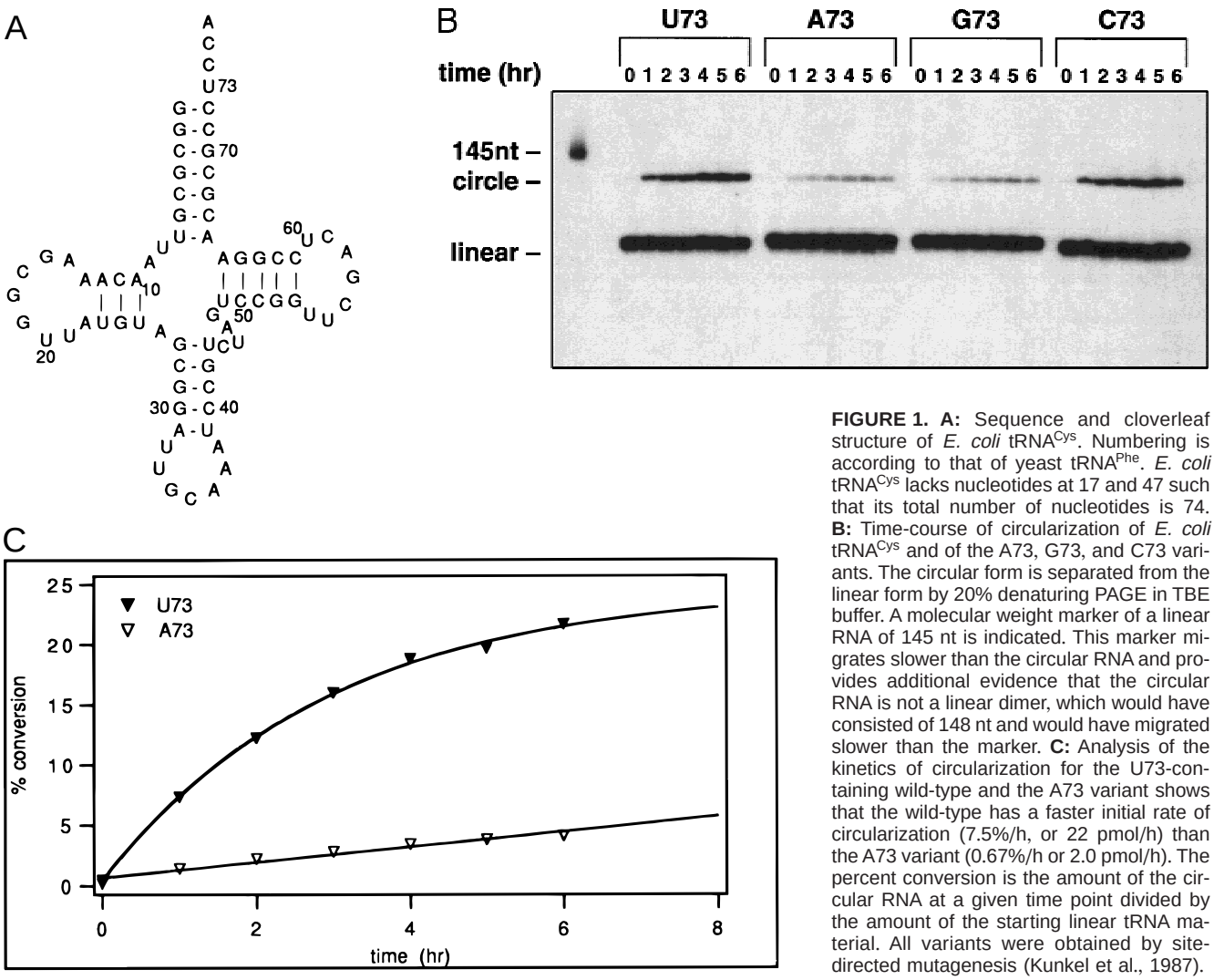

FIGURE 1. A: Sequence and cloverleaf structure of *E. coli* $tRNA^{Cys}$. Numbering is according to that of yeast $tRNA^{Phe}$. *E. coli* $tRNA^{Cys}$ lacks nucleotides at 17 and 47 such that its total number of nucleotides is 74. **B:** Time-course of circularization of *E. coli* $tRNA^{Cys}$ and of the A73, G73, and C73 variants. The circular form is separated from the linear form by 20% denaturing PAGE in TBE buffer. A molecular weight marker of a linear RNA of 145 nt is indicated. This marker migrates slower than the circular RNA and provides additional evidence that the circular RNA is not a linear dimer, which would have consisted of 148 nt and would have migrated slower than the marker. **C:** Analysis of the kinetics of circularization for the U73-containing wild-type and the A73 variant shows that the wild-type has a faster initial rate of circularization (7.5%/h, or 22 pmol/h) than the A73 variant (0.67%/h or 2.0 pmol/h). The percent conversion is the amount of the circular RNA at a given time point divided by the amount of the starting linear tRNA material. All variants were obtained by site-directed mutagenesis (Kunkel et al., 1987).

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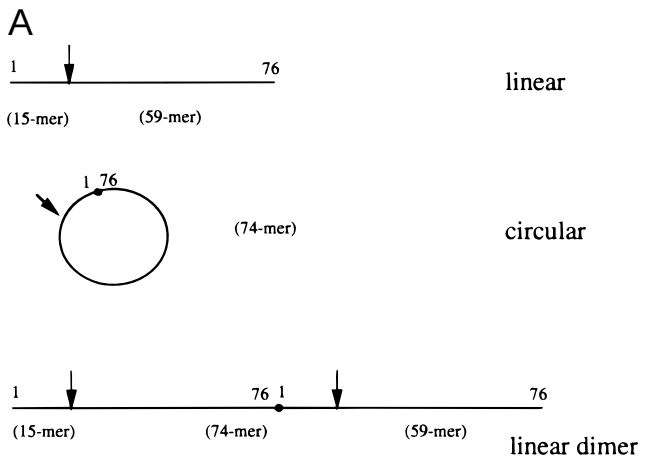
that of the linear monomer or the linear dimer (prepared by transcription of a tandom repeat of the tRNA gene). We show in Figure 2 that a site-specific cleavage targeted to G15 of the ligation product generates a fragment of the size of the full-length linear tRNA, whereas the same site-specific cleavage of a linear monomer generates two fragments and the cleavage of the linear dimer generates three fragments, one corresponding to the size of the full-length tRNA and the others corresponding to the two fragments of the linear tRNA. These patterns of cleavage are as expected. We also show that the conversion to the circular tRNA is dependent on the 5′ phosphate. A T7 transcript initiated with guanosine lacks the 5′ phosphate and is not a substrate for circularization. The dependence on the 5′ phosphate supports that circularization is catalyzed by T4 RNA ligase, because this enzyme requires a 5′ phosphate as the donor and a 3′ hydroxyl group as the acceptor (Brennan et al., 1983).

The initial rate of circularization measures the overall reaction rate that includes rates for three reversible steps. First, the ligase reacts with ATP to form a covalently adenylated enzyme intermediate. Second, the adenylate is transferred to the 5′-phosphate of the donor (pG1 in the case of *E. coli* $tRNA^{Cys}$) to form an adenylated donor. Third, a nonadenylated enzyme catalyzes the joining of the 3′ acceptor with the 5′ activated donor (Brennan et al., 1983). The differences in the initial rate most likely represent the differences in the rate of the rate-limiting step. Although the rate-limiting step is not known, we show that the differences in the initial rate appear to correlate with the predicted structure of the CCA end. For example, among the four N73 variants of *E. coli* $tRNA^{Cys}$, the fastest rate is observed with the U73-containing wild-type, which is expected to have the folded CCA end, whereas the slowest rate is observed with the A73 variant, which is expected to have an extended CCA end. This correlation suggests that the rate-limiting step might be the joining of the two ends of the tRNA. Experiments are underway to test this hypothesis.

The initial rate of circularization may also reflect (at least in part) the flexibility of the CCA end. A highly flexible CCA end that has the propensity to quickly interchange conformations can facilitate circularization. Conversely, a rigid CCA end may interfere with the



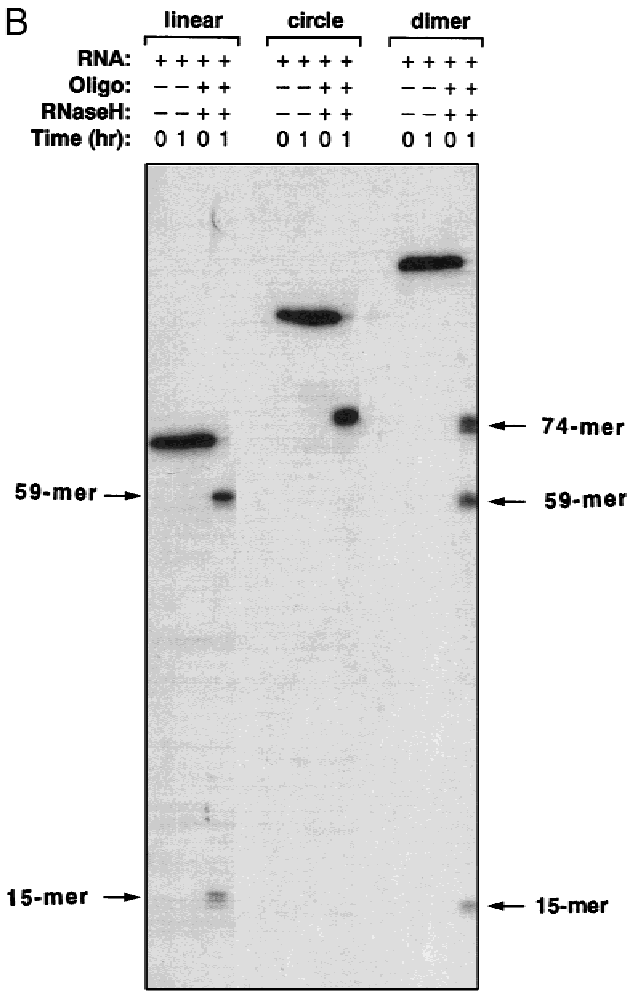


FIGURE 2. A: Strategy to distinguish among the three forms of *E. coli* $tRNA^{Cys}$, the linear monomer, the circle, and the linear dimer, by a site-specific cleavage of the tRNA. Arrows indicate the site of cleavage, which is achieved by RNase H and a 2′-O-methyl oligonucleotide (5′-TAACCGCTTTGTTAACG-3′, whereby underlined residues indicate the deoxy backbone). The cleavage is targeted to G15 of the tRNA (Xu et al., 1996; Yu et al., 1997). Cleavage of the linear tRNA (a 74-mer, indicated by nucleotides 1–76, see legend to Fig. 1) would generate two fragments (shown in parentheses; 15-mer and 59-mer, respectively); cleavage of the circular tRNA would generate one fragment (74-mer); cleavage of the linear dimer would generate three fragments (15-mer, 74-mer, and 59-mer, respectively). The small dot in the circular and linear dimer forms indicates the joining between nt 1 and 76. **B:** Analysis of the cleavage products by a 20% denaturing PAGE confirms the predicted pattern of each form. The tRNA substrate (5 pmol) was heat denatured and annealed with the oligonucleotide (20 pmol) at room temperature. Cleavage was initiated by adding 0.25 units of RNase H (Boehringer Mannheim) and continued at 37 °C for 1 h.

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ability of the ligase to perform circularization. In fact, the slower initial rate of circularization of the C1:A72/U73 variant of *E. coli* $tRNA^{Cys}$ (37.0 pmol/h) compared with that of the C1:A72/A73 variant (81.2 pmol/h) may indicate a conflict between a structure and the flexibility of the CCA end. Presumably, the presence of U73 in the C1:A72/U73 variant can restrict the flexibility of the CCA end somewhat by establishing a U73:A76 base pair. This putative base pair would not be formed in the C1:A72/A73 variant. To gain additional insight into the relationship between the rate of circularization and the structure/flexibility of the CCA end, we have initiated an NMR analysis of several acceptor stem helices of *E. coli* $tRNA^{Cys}$.

Despite the limitation of directly comparing the rate of circularization with the structure of the CCA end, our results clearly show that U73 and C1:A72 can enhance the rate of tRNA circularization. The effect of U73 is demonstrated in *E. coli* $tRNA^{Cys}$, $tRNA^{Ala}$, and yeast $tRNA^{Asp}$, whereas the effect of C1:A72 is demonstrated in *E. coli* $tRNA^{Cys}$ and previously in *E. coli* $tRNA^{fMet}$ (Bruce & Uhlenbeck, 1978). If the rate of circularization does provide an indication for the *structure* or *flexibility* of the CCA end, our results and those of others suggest that U73 or C1:A72 would confer an altered conformation of the CCA end other than that of A73, G73, or G1:C72. This raises the question of whether such an alteration can contribute to the function of the tRNA. The functional significance of C1:A72 in $tRNA^{fMet}$ during translation is well documented (Lee et al., 1992, 1993). The functional significance of U73 is also compelling. U73 is conserved in all cysteine tRNAs and is the most important nucleotide for aminoacylation of *E. coli* $tRNA^{Cys}$ by *E. coli* cysteine-tRNA synthetase (Komatsoulis & Abelson, 1993; Hamann & Hou, 1995). The contribution of U73 to the free energy of aminoacylation of *E. coli* $tRNA^{Cys}$ is by far the greatest for a single nucleotide ($\Delta\Delta G$ = 5.0–7.1 kcal/mol). It exceeds the contribution of a nucleotide to use its functional groups for making direct contact with a synthetase, which is estimated to be around 3.3 kcal/mol (Pütz et al., 1991; Hou, 1997). This suggests that some of the thermodynamic contribution of U73 to aminoacylation may derive from its ability to alter the conformation of the CCA end so as to provide a new motif for recognition by the synthetase (Puglisi et al., 1994). A similar case can be made for C73, which also facilitates tRNA circularization. C73 is the most important nucleotide for aminoacylation of *E. coli* $tRNA^{His}$ (Himeno et al., 1989). In this tRNA and other histidine tRNAs that contain C73, there is an extra G-1 that can form a base pair with C73. The extra base pair between G-1 and C73 would create an acceptor stem of eight base pairs, which may signal a structural variation that is recognized by histidine-tRNA synthetase.

U73 or C73 may also influence the ability of the CCA end to serve as a primer during replication of retroviruses. The first step of replication of the retroviral genome is the partial unfolding of the 3′ end of a tRNA to allow it to base pair with the primer binding site of the genomic RNA (Maizels & Weiner, 1994). If the CCA end adopts a fold-back structure, which captures many features of the highly stable UUCG tetraloop commonly found at the end of large RNAs (Varani et al., 1991), the stability of the structure would make it more difficult to unfold the tRNA. Alternatively, if the CCA end is highly flexible, it may not be appropriate to provide the primer binding site. Our study with antisense oligonucleotides that target various regions of *E. coli* $tRNA^{Cys}$ indicates that the UCCA sequence is not favored for primer binding. Although regions such as the anticodon stem-loop, TΨC stem-loop, and the dihydrouridine stem-loop are able to bind antisense oligonucleotides, the UCCA sequence is not readily accessible (Hou & Gamper, 1996). If the ability of the CCA end to serve as a primer during replication gives a selective advantage for the tRNA in evolution (Maizels & Weiner, 1994), then a folded or flexible structure of the CCA end is not preferred. This may partially explain why UCCA and CCCA sequences are much less frequently found than the ACCA and GCCA sequences among the present day tRNAs.

MATERIALS AND METHODS

The tRNA circularization assay

We prepared each tRNA substrate by T7 RNA polymerase and incorporated α-^{32}P-ATP during transcription (Hou, 1994). To 5 μg of each transcript, we added 0.05 μg purified tRNA nucleotidyl transferase (prepared from an over producer strain, UT481/pEC7, Cudny & Deutscher, 1986) during the last 30 min of the transcription reaction to generate homogeneous 3′ ends. Each tRNA was purified by electrophoresis on a 20% denaturing polyacrylamide gel and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 10^5 cpm/μL (~100 μM). Prior to the assay, 400 pmol of each tRNA was denatured at 80 °C for 3 min and annealed at room temperature for 5 min in a buffer (50 mM NaCl, 10 mM sodium phosphate, pH 6.5, and 0.1 mM EDTA) that was used for the NMR analysis of the fold-back structure (Puglisi et al., 1994). Circularization was initiated by adding T4 RNA ligase (Pharmacia, FPLC purified) to a final concentration of 0.375 units/μL in a reaction containing 16 μM tRNA, 50 μM ATP, 12.5% DMSO, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, and 3.2 μM of the enzyme. The threefold molar excess of ATP (50 μM) to tRNA (16 μM) was determined to give the maximal yield of the circular product. After incubation at 16 °C for various lengths of time, an aliquot was removed, loaded on a 20% denaturing PAGE in TBE buffer (89 mM Tris borate, 89 mM borate, 2 mM EDTA), and electrophoresed at 2,000 volts for 7 h. The circular tRNA migrated slower than the linear tRNA and the amount of circularization was quantitated by phosphorimage analysis.

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Initiation of T7 transcription with guanosine

A fresh stock of 200 mM guanosine was prepared by dissolving 0.34 g of guanosine (Sigma) in 6 mL of 0.33 M NaOH. An aliquot of the guanosine stock was added to a transcription reaction (pH 7.5) to a final concentration of 16 mM guanosine to give a fourfold molar excess of guanosine relative to the concentration of NTP. Transcription was initiated with T7 RNA polymerase and the product was purified by a 12% denaturing PAGE. The guanosine-initiated transcript migrated slightly slower than GMP-initiated transcript.

Synthesis of the linear dimer tRNA

We constructed a derivative of plasmid pTFMa-Cys01 (the parental plasmid containing the gene for $tRNA^{Cys}$ in plasmid pTFMa), in which the gene for *E. coli* $tRNA^{Cys}$ was duplicated in tandem. We began by converting the *Bst*N I restriction site that encodes the CCA end in the parental plasmid to an *Sma* I site. Restriction of the mutant plasmid with *Sma* I and *Bam*H I provided a linearized vector with one copy of the gene, which was joined by ligation with overlapping oligonucleotides that reconstituted the second copy of the gene. Transcription of the resulting plasmid yielded a tandem repeat of the tRNA (148 nt in length) except that the first copy of the tRNA contained a CCC end (due to the introduction of the *Sma* I site) rather than a CCA end.

Synthesis of tRNA mutants that contained C1

Mutants of *E. coli* $tRNA^{Cys}$ that contained C1 were transcribed first as a precursor that contained a 5′ extension of 9 nt (5′-GGGCAGTAA). The precursor tRNA (5 μg) was then incubated with the M1 RNA of RNase P (9 μg) to remove the 5′ extension (Altman et al., 1995). The M1 RNA was transcribed from a clone (pDW98, a generous gift of Dr. Norm Pace) by standard T7 transcription conditions. The processing by the M1 RNA was performed in a reaction containing 50 mM Tris-HCl, pH 7.6, 60 mM $MgCl_2$, 100 mM NH_4Cl, and 5% glycerol at 37 °C for 1 h, and the processed tRNA was purified by a 12% denaturing PAGE (Reich et al., 1988).

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