Permutation of a Pair of Tertiary Nucleotides in a Transfer RNA†

Ya-Ming Hou,* Therese Sterner, and Michele Jansen

Department of Biochemistry and Molecular Biology, Thomas Jefferson University, 233 South 10th Street, Philadelphia, Pennsylvania 19107

Received August 5, 1994; Revised Manuscript Received November 14, 1994®

ABSTRACT: The tertiary nucleotides at positions 15 and 48 in a tRNA establish non-Watson−Crick hydrogen interactions that connect the dihydrouridine (D) loop with the variable loop and stabilize the "L"-shaped tRNA structure. Although the majority of tRNAs have G15•C48 or A15•U48, all of the 16 possible nucleotide pairs at positions 15 and 48 can be found in the existing cytoplasmic and mitochondrial tRNA sequences. Because tRNAs contain a variety of slightly different sets of tertiary nucleotides, this complexity raises the question of whether a given tRNA sequence framework can accommodate all of the 16 compositions at positions 15 and 48. In this work, G15 and C48 in an *Escherichia coli* alanine amber suppressor tRNA were permuted, and variants were tested for biological activity *in vivo*. All but an A15•A48 variant were functional, indicating substantial flexibility at positions 15 and 48 to accommodate nucleotide variations. Analysis of the A15•A48 variant with chemical probes showed that this mutant harbors a defect that specifically changes the conformation of the anticodon sequence. Interestingly, human $tRNA^{Ala}$ has A15•A48. Additional nucleotide substitutions in *E. coli* A15•A48 $tRNA^{Ala}$ that recreate the D loop sequence of human $tRNA^{Ala}$ restored the biological activity to this tRNA by reestablishing the wild-type conformation of the anticodon sequence. The results suggest a distal relationship between the D and the anticodon loops in a tRNA and delineate covariation of specific nucleotides in the evolution of $tRNA^{Ala}$ from *E. coli* to human.

The crystal structure of yeast $tRNA^{Phe}$ established an "L"-shaped tertiary structure that consists of a coaxially stacked acceptor−TΨC stem and a D anticodon stem (Robertus *et al.*, 1974; Kim *et al.*, 1974). Formation of the L shape arises from folding of a cloverleaf secondary structure and is facilitated by base-stacking and hydrogen-bonding interactions between tertiary nucleotides that are usually conserved or semiconserved in evolution. Although the primary tRNA sequences vary considerably, the largely conserved tertiary nucleotides maintain all tRNAs in a similar three-dimensional fold (Rich & RajBhandary, 1976). This L places the amino acid attachment site and the anticodon at opposite ends of the molecule and enables tRNAs to participate in the ribosomal translational machinery during protein synthesis.

Inspection of tRNA sequences shows a complexity of different sets of tertiary nucleotides (Sprinzl *et al.*, 1991). Interpretation of this complexity is difficult. For example, many of the mammalian mitochondrial tRNAs lack the D or TΨC loops and the tertiary nucleotides therein. The bovine mitochondrial $tRNA^{Ser}$ can nonetheless fold into an "L"-like tertiary structure through alternative base pairings and other compensatory interactions (deBruijn & Klug, 1983). However, in the absence of the three-dimensional structure for this tRNA, it is difficult to predict if alternative base pairings require specific nucleotides or can be achieved by a variety of nucleotides. Previously, we have isolated a collection of bacterial tRNA mutants that functioned in protein synthesis (Hou & Schimmel, 1992). These mutants were isolated from a pool of random nucleotide substitutions in an *Escherichia coli* $tRNA^{Ala}$. Sequence analysis established that many of these mutants contained substitutions at the conserved or semiconserved positions that normally stabilize the D anticodon stem. This suggests that considerable nucleotide substitutions at some tertiary positions can be accommodated in a tRNA and that the bacterial translational apparatus, as in mammalian mitochondria (deBruijn *et al.*, 1980), can tolerate substitutions that are not normally found in the existing tRNAs.

To explore the possibility that at least some of the tertiary nucleotides in the D anticodon stem of a tRNA can be flexible, we permuted a pair of tertiary nucleotides in the D loop that contributes to the stability of the L shape and tested the capacity of the bacterial translational machinery to accommodate each mutation. We chose G15•C48 in *E. coli* $tRNA^{Ala}$ which, as defined in the structure of yeast $tRNA^{Phe}$, is a trans base pair of two hydrogen bonds that stacks on the reverse Hoogstein U8•A14 base pair in the D loop (Robertus *et al.*, 1974; Kim *et al.*, 1974). The stacking and hydrogen interactions of G15•C48 can be also achieved by A15•U48 (Klug *et al.*, 1974). This accounts for the conservation of purine 15 and its complementary pyrimidine 48 in most tRNAs. However, all 16 nucleotide variations of G15•C48 are found in the naturally occurring tRNAs (Sprinzl *et al.*, 1991), and yet no study has addressed the question of whether all 16 variations can be accommodated by a given sequence framework. In particular, while the $tRNA^{Ala}$ of *E. coli* and yeast have G15•C48, those of *Bombyx mori* and human have A15•A48 (Hou & Schimmel, 1989; Sprinzl *et al.*, 1991). Although we showed that the *B. mori* and human $tRNA^{Ala}$ are functional in *E. coli* (Hou & Schimmel, 1989) and can be aminoacylated by the *E. coli*

† This work was supported in part by Grant GM 47935 from the NIH (to Y.-M.H.) and a grant from the Lucille P. Markey Charitable Trust.

* To whom correspondence should be addressed (telephone, 215-955-4480; FAX, 215-923-9162; e-mail, hou@calvin.jci.tju.edu).

® Abstract published in *Advance ACS Abstracts,* February 15, 1995.

0006-2960/95/0434-2978$09.00/0

alanine tRNA synthetase through a common determinant in the acceptor stem (Hou & Schimmel, 1988, 1989), the effect of substituting G15•C48 with A15•A48 in the sequence framework of *E. coli* $tRNA^{Ala}$ is unknown.

In this study, all 16 variations of G15•C48 were examined in an *E. coli* alanine amber suppressor tRNA. The major determinant for the specificity of this tRNA is a G3•U70 base pair in the acceptor stem, which is outside of the anticodon (Hou & Schimmel, 1988; Park *et al.*, 1989). This means that substitution of the wild-type GGC anticodon with the amber-reading CUA anticodon retains the aminoacylation specificity of this tRNA for alanine. The amber suppressor alanine tRNA ($tRNA^{Ala/CUA}$) will not insert alanine at internal sense codons but is only used to suppress amber mutations. This enabled us to explore and delineate the structural boundaries at position 15•48 (p15•48) in the context of a tRNA that functioned in protein synthesis.

MATERIALS AND METHODS

Amber Suppressors and Test Strains. The gene for *E. coli* $tRNA^{Ala/CUA}$ was constructed in plasmid pTA-Ala. This plasmid is a derivative of pGFIB-Ala (Hou & Schimmel, 1988; Normanly *et al.*, 1986), where the synthetic tRNA gene is constructed between the *E. coli lpp* promoter and the rrnC terminator sequence. We have modified pGFIB-Ala to create pTA-Ala by introducing a synthetic bacterial phage T7 promoter in front of the tRNA gene behind the *lpp* promoter. This facilitates preparation of tRNA transcripts by T7 RNA polymerase (Samson & Uhlenbeck, 1988). We also inserted a *Bst*N1 restriction site in plasmid pTA-Ala after the CCA terminal sequence of the tRNA gene. Restriction of *Bst*N1 in pTA-Ala thus generates a DNA fragment that can be transcribed by T7 RNA polymerase to give a full-length tRNA transcript that ends in the CCA sequence. Expression of the tRNA gene by plasmid pTA-Ala *in vivo* is mediated by *lpp* promoter. Introduction of pTA-Ala to *E. coli* strain XAC-I [F′*lacI* $lacZ_{am}$ $proB^+$/F^-Δ(*lacproB*) *nalA*, *rif*, $ArgE_{am}$, *ara*] (Masson & Miller, 1986; Kleina *et al.*, 1990) or FTP3689 [F′*trpA*(UAG234)/*metB glyV*55 Δ(*tonB-trpAB17*] (Murgola & Hijazi, 1983) that harbors respectively an amber mutation in the gene for β-galactosidase or the α subunit of tryptophan synthetase conferred suppression at the amber mutation. This indicates that expression of the tRNA gene by the pTA-Ala plasmid in *E. coli* generated a functional suppressor tRNA.

Construction of tRNA Variants. Nucleotide substitutions at G15•C48 in $tRNA^{Ala/CUA}$ were achieved by site-directed mutagenesis using the mutagenesis kit of Amersham or Clonetech. Mutations were confirmed by dideoxy sequencing analysis of the entire tRNA gene in the single-stranded DNA of pTA-Ala that harbored the mutation. Plasmid pTA-Ala contains an F1 intergenic region such that, with the addition of the helper phage M13K07, single-stranded DNA can be packaged in phage and purified (Masson & Miller, 1986; Normanly *et al.*, 1986). The suppression phenotype in XAC-I was scored by the ability of a tRNA mutant to suppress the $lacZ_{am}$ mutation within 48 h on an M9 minimal plate that is supplemented with the x-gal indicator substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranose (Normanly *et al.*, 1986). The functional mutants should confer a blue color to the colonies. The suppression phenotype in FTP3689 was scored by the ability of a tRNA mutant to suppress the *trpA*(UAG234) mutation, allowing growth within 2 days on M9 minimal plates with no tryptophan supplement (no Trp) or with 0.04 μg/mL tryptophan supplement (low Trp) (Hou & Schimmel, 1988).

Diethyl Pyrocarbonate (DEPC) Modification of tRNAs. DEPC was purchased from Aldrich and used and stored according to the manufacturer's instructions. The tRNA transcripts for the DEPC modification experiment were 3′ labeled with [5′-^{32}P]pCp and T4 RNA ligase. Modification was carried out in a 100-μL total volume of 50 mM sodium cacodylate, pH 7.0, in the presence of 5 mM $MgCl_2$ (native conditions) or 0.5 mM EDTA (semidenaturing conditions). Each tRNA transcript (4 μg, 4×10^5 cpm) was reacted with 20 μL of DEPC at room temperature for 40 min with occasional vortexing (Romby *et al.*, 1987; Ehresmann *et al.*, 1987). The modified tRNAs were then precipitated with ethanol, washed once, and precipitated again. The tRNA pellets were dissolved in 50 μL of aniline−acetic acid (pH 5.0) and incubated at 60 °C to induce strand scissions at the site of modifications. The cleavage products were dried, dissolved in 20 μL of H_2O, ethanol precipitated twice, and analyzed by denaturing polyacrylamide (12%) gel electrophoresis and autoradiography.

Dimethyl Sulfate (DMS) Modification of tRNAs. DMS was purchased from Fluka and used according to the manufacturer's instructions. Modification with DMS was achieved with 4 μM tRNAs that were 3′ end labeled with [5′-^{32}P]pCp and T4 RNA ligase. The modification reaction contained 0.5% DMS and was investigated under native (10 mM $MgCl_2$, 50 mM sodium cacodylate, pH 7.2) or semidenaturing conditions (1 mM EDTA, 50 mM sodium cacodylate, pH 7.2) for 5 min at room temperature. After reaction with DMS, tRNAs were treated with aniline as described in the procedure for DEPC modification. The chain-scissioned fragments were separated by gel electrophoresis and detected by autoradiography (Hou *et al.*, 1993).

RESULTS

All variants of *E. coli* $tRNA^{Ala/CUA}$ at G15•C48 were tested for their ability to suppress the amber mutations at $lacZ_{am}$ of XAC-I and at *trpA*(UAG234) of FTP3689. Suppression of these two amber mutations requires insertion of different amino acids and at different efficiencies. The $lacZ_{am}$ mutation can be suppressed by insertion of any amino acids such that any tRNA which productively interacts with the components in the translational machinery will confer a sup^+ phenotype (Kleina *et al.*, 1990; Hou & Schimmel, 1992). This includes interactions with an aminoacyl tRNA synthetase, the elongation factors, and the ribosomes. In contrast, the codon context of *trpA*(UAG234) is more stringent; only tRNAs that are charged with alanine or glycine confer the sup^+ phenotype (Murgola & Hijazi, 1983). Additionally, suppression of *trpA*(UAG234) is achieved with tRNA suppressors that have higher suppression efficiencies than those required for $lacZ_{am}$. We define the suppression efficiency of a tRNA as the fraction of ribosomes that were enabled to read through at the amber mutation over the total attempts of the ribosomes. We have previously shown that the starting $tRNA^{Ala/CUA}$ retains the specificity for alanine and has 20% suppression efficiency relative to the native $tRNA^{Ala}$ (Hou & Schimmel, 1988). This wild-type alanine amber suppressor is a functional suppressor at both $lacZ_{am}$ and *trpA*(UAG234) (Table 1).

Table 1: Suppression of All Variants at Positions 15 and 48 of *E. coli* $tRNA^{Ala/CUA}$ [a]

nucleotide substitutions		suppression at $lacZ_{am}$	suppression at *trpA*(UAG234)		proposed no. of hydrogen bonds
p15	p48		low Trp	no Trp	
G	C	+	+	+	2
	A	+	−	−	1
	U	+	+	+	2
	G	+	+	−	2
C	C	+	+	±	2
	A	+	±	−	2
	U	+	+	±	2
	G	+	−	−	2
A	C	+	+	+	2
	A	−	−	−	2
	U	+	±	−	2
	G	+	±	−	1
U	C	+	+	+	2
	A	+	+	+	2
	U	+	+	+	2
	G	+	+	+	2

[a] The wild type and 15 nucleotide variations created at G15·C48 of *E. coli* $tRNA^{Ala}$ are given. Each was tested and scored for suppression at $lacZ_{am}$ by (+) (blue streak) or (−) (white streak) and at *trpA*(UAG234) by (+) (growth within 2 days), (−) (no growth in 2 days), or (±) (weak growth as streaks but no single colonies in 2 days). The proposed number of hydrogen bonds for each variation at p15·48 is the maximum under trans-pairing configuration.

With the exception of the A15·A48 variant, all variants were functional at $lacZ_{am}$. This suggests that the majority of these variants were aminoacylated and were able to participate in protein synthesis. Model building studies indicated that most of the substitutions at p15·48 have the potential of making a two-hydrogen-bonded tertiary interaction (Table 1). Here, we proposed for each variant a tertiary interaction at p15·48 that will achieve the maximal potential of hydrogen bonds while maintaining the *trans* configuration between positions 15 and 48 (not shown). Our analysis suggested that, as with the majority of the tRNA mutants, the A15·A48 mutant could form a two-hydrogen-bonded tertiary interaction. In contrast, the G15·A48 and A15·G48 mutants would have at most one hydrogen bond. Yet the G15·A48 and A15·G48 variants were sup^+ at $lacZ_{am}$ whereas the A15·A48 variant was sup^-. It appears that there is no direct relationship between the suppression pattern and the proposed number of hydrogen bonds. Further, investigation of the proposed hydrogen bonds showed that many would distort the phosphodiester backbone of the wild-type base pair at G15·C48. The functional phenotype of these variants (except for the A15·A48 variant) demonstrated the ability of the tRNA sequence framework to accommodate considerable variations of hydrogen interactions at p15·48.

The *trpA*(UAG234) amber mutation eliminated the ability of *E. coli* cells to synthesize tryptophan synthetase (Murgola & Hijazi, 1983). Cells that harbored this mutation required supplementation of tryptophan at 20 μg/mL to grow. Without tryptophan supplement, 9 of the 16 tRNAs had the Trp^+ phenotype, indicating suppression of the *trpA*(UAG234) amber mutation. These 9 tRNAs presumably retain the specificity for alanine. There were six variants that were lac^+ but Trp^-. These could be misacylated with an amino acid other than alanine or could be relatively inefficient suppressors compared to the wild type. With tryptophan supplementation at a suboptimal 0.04 μg/mL (low Trp, Table 1), four of the six variants became Trp^+. The switch from Trp^- to Trp^+ as the tryptophan concentration increased suggests that these four variants were capable of inserting alanine to an amber codon but that their suppression efficiencies were weak for the context of *trpA*(UAG234). Addition of a suboptimal concentration of tryptophan complemented their defect in suppression efficiency and conferred the ability to suppress *trpA*(UAG234). Among the 13 lac^+Trp^+ tRNAs, we cannot rule out the possibility that some might be misacylated with glycine.

The remaining two lac^+Trp^- mutants, G15·A48 and C15·G48, were prepared *in vitro* by T7 RNA polymerase and tested for aminoacylation with alanine. Compared to the wild-type suppressor, the catalytic efficiency of aminoacylation of the G15·A48 and C15·G48 variants is decreased by a factor of 2 and 4, respectively. This level of decrease is not sufficient to eliminate the ability of these tRNAs to be aminoacylated with alanine *in vivo*. Earlier studies have shown that substitutions of the G3·U70 determinant, which eliminated the alanine identity of $tRNA^{Ala/CUA}$ *in vivo*, decreased the catalytic efficiency by a factor of 10^6 or more (Park *et al.*, 1989). It is most likely that the failure of the G15·A48 and C15·G48 variants to suppress *trpA*(UAG234) was not due to misacylation but due to a much weaker suppression efficiency. We determined the suppression efficiencies of the G15·A48 and C15·G48 mutants by measuring the activity of the $lacZ_{am}$ gene product. This analysis showed that the G15·A48 mutant is 10% as efficient as the wild-type suppressor while the C15·G48 mutant is only 2% as efficient. The significantly reduced suppression efficiency for the C15·G48 mutant was unexpected. This mutant contains the reciprocal of the wild-type G15·C48 base pair and is capable of maintaining similar hydrogen interactions at p15·48. The effect of C15·G48 substitution on the structure of the tRNA remains to be determined.

We investigated the A15·A48 variant in more detail because of its lack of suppression at both the $lacZ_{am}$ and *trpA*(UAG234) mutations. Aminoacylation of the A15·A48 transcript showed that it retains the specificity for alanine and has a catalytic efficiency 8-fold lower than that of the wild type. To test the possibility that this mutant may not be stably synthesized in *E. coli* so that it was unable to suppress the amber mutations, we examined the presence of A15·A48 $tRNA^{Ala/CUA}$ in total tRNAs. We and others have previously demonstrated that a plasmid-encoded tRNA, if stable inside the cells, should be readily detectable from all tRNAs when separated by a nondenaturing polyacrylamide gel electrophoresis (12%) (Seong & RajBhandary, 1987; Hou & Schimmel, 1988). The principle of this separation is that the mobility of the different tRNAs will be approximately in accordance with their GC content. Figure 2 shows that the wild-type $tRNA^{Ala/CUA}$ species is readily detected by this technique because of the large amounts produced from the multicopy plasmid. Similarly, the A15·A48 variant is easily visualized at an intensity that is virtually identical to that of the wild-type tRNA. On the other hand, the control tRNAs which were prepared from an *E. coli* strain that did not harbor a plasmid-encoded tRNA gene show no overexpressed tRNA species. We note that the wild type and the A15·A48 variant migrate to the same position on the gel in Figure 2. This indicates that the A15·A48 variant is not only stably synthesized but also properly processed as is the wild-type suppressor.

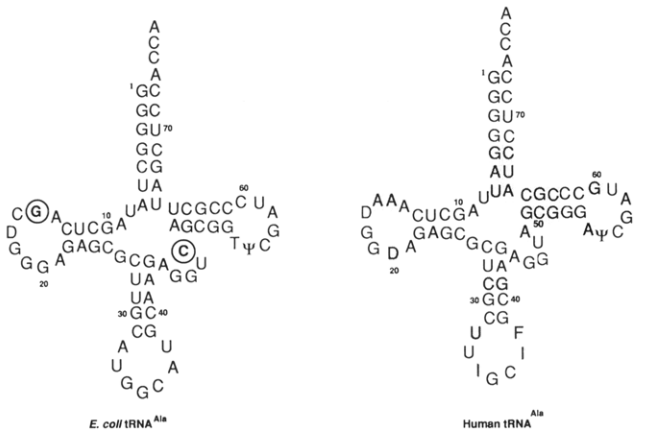

FIGURE 1: Sequence and cloverleaf structure of *E. coli* $tRNA^{Ala/GGC}$ and of human $tRNA^{Ala/IGC}$. The amber suppressor *E. coli* $tRNA^{Ala/CUA}$ was created by substituting the GGC anticodon sequence of the wild type with the amber-reading CUA sequence. The G15•C48 base pair in *E. coli* $tRNA^{Ala}$ is circled, and nucleotides in human tRNA that are different from their *E. coli* counterpart are shaded.

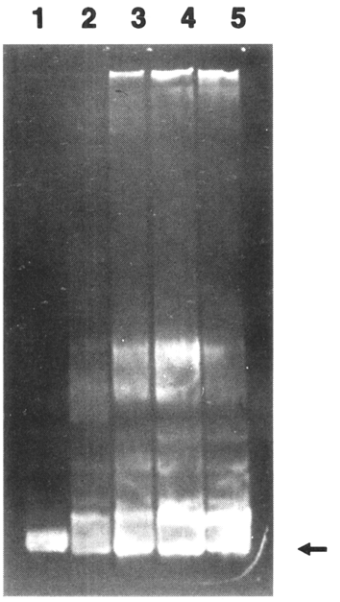

FIGURE 2: Analysis of stable synthesis of mutant $tRNA^{Ala/CUA}$ by a 12% nondenaturing gel electrophoresis. The purified transcript of *E. coli* $tRNA^{Ala/CUA}$ (15 μg) is shown in lane 1. Equal amounts of the total tRNA (40 μg) from XAC-I cells (lane 2) and from those harboring the plasmid-encoded wild-type $tRNA^{Ala/CUA}$ (lane 3), the A15•A48 variant (lane 4), and the A16U20/A15•A48 $tRNA^{Ala/CUA}$ (lane 5) are shown. The arrow indicates the migration of the overexpressed wild-type $tRNA^{Ala/CUA}$, the A15•A48 mutant, and the A16U20/A15•A48 revertant in lanes 3−5, respectively.

The A15•A48 mutant shares the nucleotide composition of A15•A48 with the human and *B. mori* alanine tRNAs that are functional in *E. coli*. This suggests that the A15•A48 substitution *per se* does not prevent interactions with specific components in the bacterial translational apparatus. Inspection of the human or *B. mori* $tRNA^{Ala}$ shows that A15•A48 is accompanied by unusual nucleotides at other conserved positions (Figure 1). For example, in human and *B. mori* alanine tRNAs, the normally conserved T54 and semiconserved pyrimidine 60 in the TΨC stem are replaced respectively by A54 and purine 60 in human $tRNA^{Ala}$. This suggests that the defect in *E. coli* A15•A48 $tRNA^{Ala/CUA}$ may be due to the structural incompatibility of A15•A48 with the sequence framework of this tRNA. We sought to alleviate this defect by recreating structural features in human $tRNA^{Ala}$ that are absent in *E. coli* A15•A48 $tRNA^{Ala/CUA}$. Substitutions of T54 with A54, and of C60 with G60, did not restore the function of the *E. coli* A15•A48 variant. However, introduction of A16 and U20 in the D loop that recreated the D loop sequence of the human and *B. mori* $tRNA^{Ala}$ enabled this variant to suppress the amber mutation at $lacZ_{am}$.

The A16U20 revertant was synthesized in *E. coli* to the same level as those of the wild-type suppressor and the A15•A48 variant (Figure 2). The suppression efficiency of this revertant at $lacZ_{am}$ is nearly the same as that of the wild type (90% of the wild type). However, this A16U20 revertant failed to suppress the amber mutation at *trpA*(UAG234). We tested the ability of this revertant to be aminoacylated with alanine *in vitro* and showed that its catalytic efficiency of aminoacylation was decreased from the wild type by a factor of 32. Thus, this A16U20 revertant was aminoacylated with alanine 4-fold less well than the original A15•A48 mutant. This decrease in the catalytic efficiency of aminoacylation apparently afforded suppression at $lacZ_{am}$ but not at *trpA*(UAG234). It is possible that the sequence framework of this revertant may have created recognition elements for other aminoacyl tRNA synthetases such that charging with alanine is outcompeted *in vivo*, resulting in misacylation that cannot suppress *trpA*(UAG234).

We analyzed the A15•A48 variant and the A16U20 revertant with the chemical probes DEPC and DMS to delineate the structural basis of their different phenotypes in protein synthesis. DEPC primarily modifies the N7 of A residues but can react with other bases under our reaction conditions. Under native (with Mg^{2+}) or semidenaturing (without Mg^{2+}) conditions, base modification by DEPC, followed by treatment with aniline, led to cleavage of the sugar−phosphate blackbone in the tRNA. The cleavage products were separated by a denaturing gel, and the sites of cleavage were determined by comparing the lengths of the cleavage products with an RNA ladder. We compared in Figure 3A the DEPC-generated cleavage products of the wild-type *E. coli* $tRNA^{Ala/GGC}$ with those of the A15•A48 $tRNA^{Ala/GGC}$ variant and the A16U20/A15•A48 $tRNA^{Ala/GGC}$ revertant. We also tested DEPC modification using the amber suppressor $tRNA^{Ala/CUA}$ as the wild type and comparing it with the A15•A48 variant and the A16U20 revertant in the seqeunce framework of $tRNA^{Ala/CUA}$. The results were similar (data not shown). Figure 3A shows that, within the range of the detection, the A15•A48 variant primarily differed from the wild type in the D stem and the anticodon loop. Specifically, A23/G24 in the D stem and A37A38 in the anticodon loop reacted favorably in the A15•A48 variant but did not react with DEPC in the wild type. This indicates an alteration of the D stem and anticodon loop in the A15•A48 variant that allowed accessibility of A23/G24 and A37A38 to DEPC. Because A37A38 are immediately adjacent to the anticodon sequence and are usually stacked with the anticodon (Rich & RajBhandary, 1976), the accessibility of A37A38 to DEPC also implied perturbation of the stacking interactions with the anticodon. However, the reactivity of A37A38 with DEPC in the A16U20 revertant was significantly reduced, indicating partial restoration of the stacking interactions with the anticodon. Additionally, the revertant also diminished its reaction with DEPC at A23/G24, although its level of reactivity was still stronger than that in the wild type.

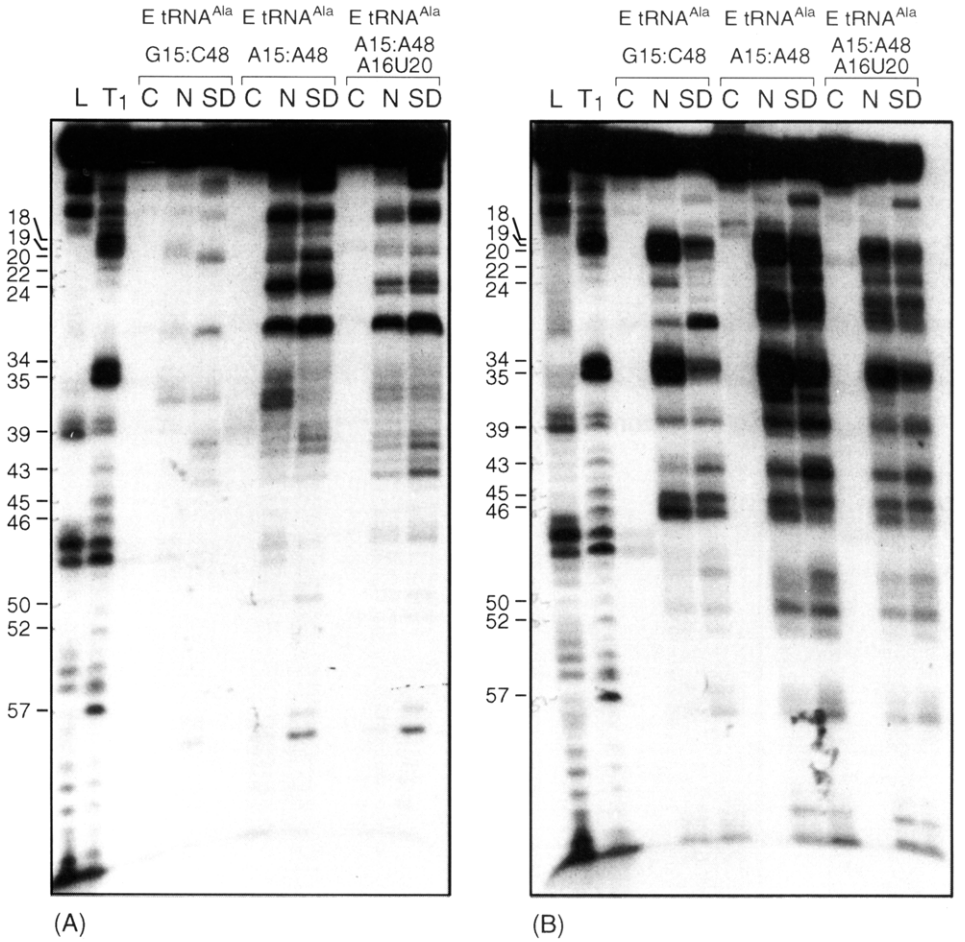

FIGURE 3: Chemical modification of the wild-type *E. coli* tRNA$^{Ala/GGC}$ and the variants A15•A48 tRNA$^{Ala/GGC}$ and A15•A48/A16U20 tRNA$^{Ala/GGC}$ with (A) DEPC and (B) DMS. Each tRNA was prepared as a transcript by the T7 RNA polymerase and was reacted with the respective chemical probe under native (N) or semidenaturing (SD) conditions. A control (C) of no chemical treatment was included for each tRNA. The L and T_1 lanes show respectively the alkali hydrolysis and the T_1 digestion of the wild-type tRNA. The numbers on the left of each gel indicate the positions of T_1-digested fragments.

Analysis with DMS of the wild type, the A15•A48 variant, and the A16U20 revertant is shown in Figure 3B. DMS reacts with the N7 of G residues that are not involved in stacking or tertiary interaction. Here we show that the A15•A48 variant differed from the wild type largely in the intensity of reactivity from G18 in the D loop to G35 in the anticodon sequence. Compared to the wild type, the A15•A48 variant had enhanced reactivity at G26 and G30 in the D anticodon stem and at G34G45 in the anticodon sequence. Although DMS did not favorably react with G24 in the A15•A48 variant as in the DEPC analysis, it attacked the adjacent G26 and G30 in the anticodon stem, suggesting perturbation of the anticodon stem as well. Thus, the integrity of both the D and anticodon stems of the tRNA is affected by the A15•A48 substitution. As with the DEPC analysis, the preferential reactivity of the A15•A48 variant with DMS at the anticodon sequence indicates disruption of the anticodon stacking and suggests a conformational change of the anticodon triplet from a stacked conformation in the wild type to a unstacked conformation in the A15•A48 variant. This conformational change in the anticodon loop appeared to be suppressed by the introduction of A16U20 in the revertant as the reactivity of G34G35 to DMS was reduced to that of the wild type. Interestingly, G24 and G30 in the revertant remained accessible to DMS.

Taken together, the results of DMS and DEPC analyses showed that substitution of G15•C48 with A15•A48 in *E. coli* tRNA^{Ala} disturbed the structure of the D anticodon stem and conferred a conformational change in the anticodon loop, including the anticodon triplet. Introduction of A16U20 to the A15•A48 variant switched the conformation of the anticodon loop to that similar to the wild type. Although this A16U20 substitution did not completely restore the wild-type structure in the D anticodon stem, the restored anticodon conformation provides a plausible explanation for its functional phenotype.

DISCUSSION

Our results show that the sequence framework of *E. coli* tRNA^{Ala} can accommodate all but one nucleotide variation at G15•C48. Although these variations include alterations of hydrogen-bonding and stacking interactions, they do not perturb the ability of the tRNA to participate in protein synthesis. The only nonfunctional substitution is A15•A48 which, based on our results, induces a conformational change in the anticodon loop that may interfere with the stacking of the anticodon sequence. Additional substitutions of A16U20 in the D loop, which restore stacking at least in part to the anticodon sequence, also restore the biological function of the A15•A48 variant. Thus, the conformational change in the anticodon loop largely accounts for the inability of the A15•A48 variant to participate in protein synthesis. Although substitution of G15•C48 with A15•A48 is localized to the D−V loops, which is at the corner of the L-shaped tRNA

structure, we suggest a distal structural relationship between this part of the molecule and the anticodon loop. Consistent with this view, earlier work by Hirsh showed that alteration in the D stem had a structural effect on the anticodon sequence of *E. coli* $tRNA^{Trp/CCA}$ (Hirsh, 1971). This alteration involved replacing the wild-type U11•G24 base pair in the D stem with a U11•A24 base pair. The enhanced stability in the D stem apparently changed the conformation of the anticodon such that it was able to read an opal UGA codon through an unusual A•C pairing in the third codon position.

A recent study of *E. coli* $tRNA^{Trp/CUG}$, which has an anticodon sequence $^{3'}$GUC, has provided additional support for the distal relationship between the structure of the D anticodon stem and the conformation of the anticodon (Schultz & Yarus, 1994a,b). To suppress the UAG amber mutation, this tRNA needs a normally unacceptable G•U wobble at the first codon position and a C•A mismatch at the third codon position. Through saturating mutagenesis of the anticodon stem of the tRNA, the majority of the variants that enhanced UAG suppression were found to contain nucleotide substitutions at p27•43 located at the top base pair of the anticodon stem. In particular, mismatches at p27•43 in general had better efficiency of suppression than paired nucleotides. This suggests that alteration of the tRNA structure, possibly through disruption of the p27•43 base pair, can induce conformational change in the anticodon to confer unusual pairing activity. In the tRNA L shape, p27•43 is adjacent to p15•48 in the structure. It is conceivable that they may exert a similar structural effect on the anticodon conformation. In the case of *E. coli* $tRNA^{Trp/CUG}$, we cannot rule out that such a structural effect may be indirectly mediated through ribosomes to accommodate unusual codon–anticodon pairing.

In principle, A15 and A48 can form a tertiary interaction that involves the N1 as the hydrogen acceptor and the 6-amino group as the hydrogen donor. It is possible that this tertiary interaction alters the backbone conformation at p15•48 from that of the G15•C48 base pair of the wild type. Thus, by virtue of a conformational change in the D loop, the A15•A48 substitution induces a conformational change in the anticodon loop. There is evidence that suggests a different D loop conformation of the A15•A48 mutant from that of the wild type. For example, the A15•A48 mutant shows less cooperativity during melting analysis and has a 10-fold reduced rate of Pb cleavage in the D loop (Jansen and Hou, unpublished results). The latter analysis evaluates the structural integrity of the Pb binding site in the D–TΨC loops (Krzyzosiak *et al.*, 1988; Behlen *et al.*, 1990). The reduction in the cleavage rate of the A15•A48 variant is comparable to that of substitutions of specific nucleotides in the Pb binding site and is an indication of structural alteration in the D loop.

Functional compensation of A15•A48 appears to require specific nucleotide substitutions in the D loop. While A16U20 was successful in restoring activity to the A15•A48 mutant, substitution of C13A21 with G13U21 was not. The implication is that A16U20 leads to yet another conformational change in the D loop that ultimately is transmitted to the anticodon loop. The A16U20 revertant has the same sequence in the D–V loops as that in the $tRNA^{Ala}$ of human and *B. mori*. This suggests that, in order to preserve the function of a tRNA in protein synthesis, evolution of $tRNA^{Ala}$ requires that the change of G15•C48 to A15•A48 from *E. coli* to higher mammalian cells be accompanied by the change of C16G20 to A16U20.

Although the A16U20 substitution suppresses the conformational change in the anticodon loop in the A15•A48 variant, it does not completely restore the conformation to that of the wild type. If the anticodon loop conformation of the A16U20/A15•A48 mutant is an indication of the anticodon loop conformation of the human and *B. mori* alanine tRNAs, this will suggest that the mammalian tRNAs differ from *E. coli* $tRNA^{Ala}$ in the anticodon loop. Our analysis of the effect of nucleotide substitutions at p15•48 on the anticodon loop conformation of *E. coli* $tRNA^{Ala}$ is based on phenotypes of tRNA suppressors that are overproduced *in vivo* and on structural probing of tRNA transcripts that are synthesized *in vitro*. While tRNA transcripts contain no modified nucleotides, the extent of base modification of overproduced tRNA suppressors is unknown. We have not used tRNA suppressors that are purified from cells for structural analysis with DEPC or DMS. We therefore cannot address the role of base modification in anticodon conformation change. Nonetheless, the effect of base modification is likely small, because the naturally occurring *E. coli* $tRNA^{Ala/GGC}$ contain nucleotide modifications at D17, T54, and Ψ55 and none in the anticodon loop (Sprinzl *et al.*, 1991). The lack of modification in *E. coli* $tRNA^{Ala}$ in the anticodon loop is in contrast to the extensively modified anticodon loop of mammalian $tRNA^{Ala}$'s. This may further enhance the structural differences in the anticodon loop between the *E. coli* and mammalian tRNAs.

ACKNOWLEDGMENT

We thank Drs. H. Gamper, K. Musier-Forsyth, and P. Schimmel for discussion and critically reading the manuscript.

REFERENCES

Behlen, L. S., Sampson, J. R., DiRenzo, A. B., & Uhlenbeck, O. C. (1990) *Biochemistry 29*, 2515–2523.

deBruijn, M. H. L., & Klug, A. (1983) *EMBO J. 2*, 1309–1321.

deBruijn, M. H. L., Schreier, P. H., Eperon, J. C., Barrell, B. G., Chen, E. Y., Armstrong, P. W., Wong, J. F. H., & Roe, B. A. (1980) *Nucleic Acids Res. 8*, 5213–5222.

Ehresmann, C., Baudin, F., Mougel, M., Romby, P., Ebel, J.-P., & Ehresmann, B. (1987) *Nucleic Acids Res. 15*, 9109–9128.

Hirsch, D. (1971) *J. Mol. Biol. 58*, 439–458.

Hou, Y.-M. (1994) *Biochemistry 33*, 4677–4681.

Hou, Y.-M., & Schimmel, P. (1988) *Nature 333*, 140–144.

Hou, Y.-M., & Schimmel, P. (1989) *Biochemistry 28*, 6800–6804.

Hou, Y.-M., & Schimmel, P. (1992) *Biochemistry 31*, 4157–4160.

Hou, Y.-M., Westhof, E., & Giege, R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6776–6780.

Kim, S. H., Suddath, F. L., Quigley, G. J., McPherson, A., Sussman, J. L., Wang, A. H. J., Seeman, N. C., & Rich, A. (1974) *Science 185*, 435–440.

Kleina, L. G., Masson, J.-M., Normanly, J., Abelson, J., & Miller, J. H. (1990) *J. Mol. Biol. 213*, 705–717.

Klug, A., Ladner, J., & Robertus, J. D. (1974) *J. Mol. Biol. 89*, 511–516.

Krzyzosiak, W. J., Marciniec, T., Wiewiorowski, M., Romby, P., Ebel, J. P., & Giege, R. (1988) *Biochemistry 27*, 5771–5777.

Masson, J.-M., & Miller, J. H. (1986) *Gene 47*, 179–183.

Murgola, E. J., & Hijazi, K. A. (1983) *Mol. Gen. Genet. 191*, 132–137.

Normanly, J., Odgen, R. C., Harvath, H. J., & Abelson, J. (1986) *Nature 321*, 213–219.

Park, S. J., Hou, Y.-M., & Schimmel, P. (1989) *Biochemistry 28*, 2740–2746.

Rich, A., & RajBhandary, U. L. (1976) *Annu. Rev. Biochem. 45*, 805–860.

Robertus, J. D., Ladner, J. E., Finch, J. T., Thodes, D., Brown, R. D., Clark, B. F. C., & Klug, A. (1974) *Nature 250*, 546–551.

Romby, P., Moras, D., Dumas, P., Ebel, J. P., & Giege, R. (1987) *J. Mol. Biol. 195*, 193–204.

Sampson, J. R., & Uhlenbeck, O. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1033–1037.

Schultz, D. W., & Yarus, M. (1994a) *J. Mol. Biol. 235*, 1381–1394.

Schultz, D. W., & Yarus, M. (1994b) *J. Mol. Biol. 235*, 1395–1405.

Seong, B. L., & RajBhandary, U. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 334–338.

Sprinzl, M., Dank, N., Nock, S., & Schon, A. (1991) in *compilation of tRNA and tRNA gene sequences*, Laboratory of Biochemistry, University of Bayreuth.

BI941800N