Recognition of $tRNA^{Cys}$ by *Escherichia coli* Cysteinyl-tRNA Synthetase†

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Received January 25, 1993; Revised Manuscript Received May 5, 1993

ABSTRACT: A study of the recognition of $tRNA^{Cys}$ by *Escherichia coli* cysteinyl-tRNA synthetase using *in vivo* and *in vitro* methods was performed. All three anticodon nucleotides, the discriminator nucleotide (73), and some elements within the tertiary domain (the D stem/loop, the TΨC stem/loop, and the variable loop) are important for recognition; the anticodon stem and acceptor stem appear to contain no essential elements. A T7 RNA polymerase transcript corresponding to $tRNA^{Cys}$ is only a 5.5-fold worse substrate than native $tRNA^{Cys}$ (in terms of the specificity constant, k_{cat}/K_m), mainly due to an increase in the value of K_m for the transcript. The greatest loss of specificity caused by mutation of a single nucleotide occurs when the discriminator U73 is changed; k_{cat}/K_m declines 3–4 orders of magnitude depending on the substitution. Mutations in the wobble nucleotide of the anticodon also cause reductions in the specificity constant of 3 orders of magnitude, while mutations in the other anticodon nucleotides caused lesser effects. Interestingly, a C35A mutation (with the phenylalanine anticodon GAA) had no effect on aminoacylation by the cysteinyl-tRNA synthetase. Several amber suppressor tRNAs were constructed whose *in vivo* identity did not correlate with their *in vitro* specificity, indicating the need for both types of experiments to understand the factors which maintain tRNA specificity.

The accurate conversion of genes into their physical manifestation as proteins is critically dependent upon the fidelity of translation. Translational accuracy requires that aminoacyl-tRNA synthetases maintain a high degree of discrimination between cognate and noncognate tRNA species. The challenge of such discrimination is formidable since all tRNAs appear to share a certain degree of structural similarity, presumably imposed by the requirement that they interact with the ribosome and elongation factors. Furthermore, although some synthetases (e.g., the cysteinyl-tRNA synthetase of *Escherichia coli*) have only one cognate tRNA, others must recognize multiple tRNA species known as isoacceptors (Komine et al., 1990; Sprinzl et al., 1991), which vary in sequence.

To date, the most general observation of studies of tRNA recognition is that the elements which are used by synthetases to distinguish tRNAs are individualistic [for review see Schulman (1991); Normanly and Abelson (1989); Schimmel (1987)]. The obvious solution to recognizing tRNAs is for the synthetase to interact with the anticodon nucleotides, and in fact many synthetases do this. The paradigm for such recognition is the methionyl-tRNA synthetase (Schulman & Pelka, 1983; Schulman & Pelka, 1988). However, most anticodon recognizing synthetases also recognize elements outside of the anticodon as well. Some synthetases do not utilize the anticodon at all, such as the alanyl- (Hou & Schimmel, 1988; Park et al., 1989) and seryl- (Himeno et al., 1990; M. Saks and J. Sampson, personal communication) tRNA synthetases of *E. coli*. In many organisms the prime determinant of alanine identity is a single G-U base pair at position 3:70 in the acceptor stem (Hou & Schimmel, 1989b; McClain et al., 1988; McClain & Foss, 1988; McClain et al., 1991; Musier-Forsyth et al., 1991). Results of both *in vivo* and *in vitro* experiments indicate that the seryl-tRNA synthetase of *E. coli* uses a broader set of elements to define cognate tRNAs, including the long extra loop of type II tRNAs and nucleotides in the acceptor and D stems (*in vivo* experiments, Normanly et al. (1986); Normanly et al. (1992); *in vitro* experiments, Himeno et al. (1990); J. Sampson and M. Saks, personal communication).

In *E. coli*, there is a single cysteine-accepting tRNA (Figure 1A) with the anticodon GCA which recognizes two codons, UGC and UGU. The *E. coli* cysteinyl-tRNA synthetase gene was originally characterized by Bohman and Isaksson (1979) and has recently been cloned and sequenced by two different laboratories. The sequence indicates that the monomer unit is a peptide of 461 amino acids with a molecular weight of 52 kDa (Hou et al., 1991; Eriani et al., 1991). The CysRS[1] is a synthetase of type I. Such synthetases are characterized by two conserved sequence motifs, HIGH and KMSKS, which are indicative of the Rossman fold, an ATP binding domain [Eriani et al., 1990; reviewed in Moras (1992)].

A combined *in vivo/in vitro* study of tRNA recognition was undertaken to investigate the relationship between the identity and recognition sets for an aminoacyl-tRNA synthetase, and the cysteine system in *E. coli* was well suited to this task. Previous work by Normanly et al. (1986b) indicated that an amber suppressor $tRNA^{Cys}$ retained cysteine identity, making it possible to do *in vivo* experiments. A cysteine-specific amber suppression assay (Michaels et al., 1990; C. W. Kim and J. Miller, personal communication) based on a thymidylate synthetase gene with an amber mutation at the active site was available for rapid determination of cysteine acceptance *in vivo* [see also Dev et al. (1988)]. For *in vitro* experiments, an *E. coli* strain which overproduces the cysteinyl-tRNA synthetase was available (Eriani et al., 1991; Hou et al., 1991).

† Supported by N.I.H. Grant No. GM32637-10 to J.N.A. and N.I.H. training Grant No. 5 T32 GM07616 to G.A.K.

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[1] Abbreviations: CysRS, cysteinyl-tRNA synthetase; DHFR, dihydrofolate reductase; DTT, dithiothreitol; EDTA, Na_2 ethylenediamine tetraacetate; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; GlnRS, glutaminyl-tRNA synthetase; NTP, nucleotide triphosphate; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TBE, Tris–borate–EDTA electrophoresis buffer; TCA, trichloroacetic acid; Tris, tris(hydroxyethyl)aminomethane.

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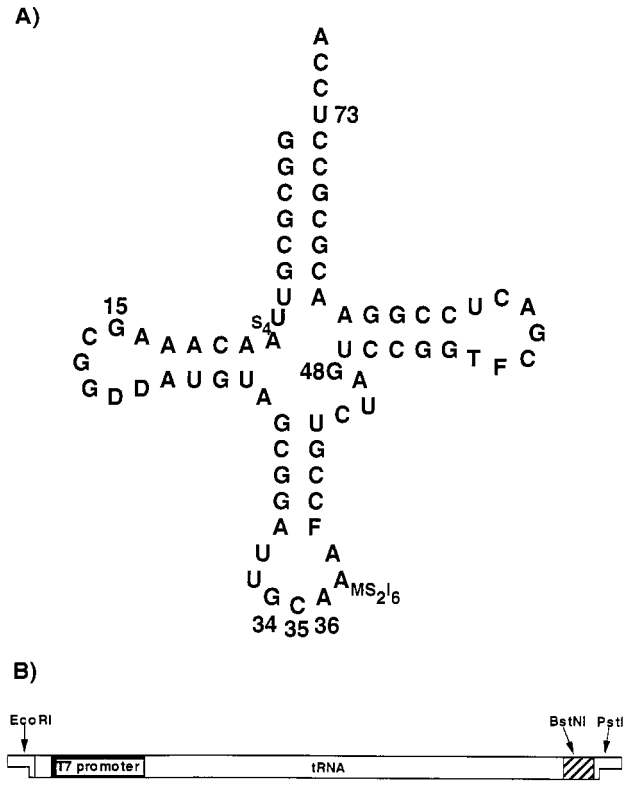

FIGURE 1: Cloning and expression strategy for mutants of $tRNA^{Cys}$. (A) RNA sequence of $tRNA^{Cys}$ from *E. coli* [sequence from Sprinzl et al. (1991)]. (B) Standard cassette produced from six oligonucleotides for cloning tRNAs. See text for details of the cloning.

We therefore conducted *in vivo* and *in vitro* studies of the recognition of *E. coli* cysteine tRNA and have determined several elements of the recognition and identity sets. Evidence is presented that the anticodon nucleotides, as well as nucleotide 73 (the discriminator base), play important roles in cysteine tRNA recognition. We further demonstrate that some elements or structural features within the tertiary domain (comprising the D stem, TΨC stem, and variable loop region) affect recognition by CysRS and that there are apparently no such elements in either the anticodon stem or the acceptor stem. Finally, evidence will be presented that either *in vivo* or *in vitro* data must be interpreted cautiously in the absence of the other.

MATERIALS AND METHODS

Strains and Plasmids. Strain XAC-1 is F′ $lacI_{373}$ $lacZ_{u118am}$ $proB^+/\Delta(lac\ pro)_{xIII}$ $argE_{am}$ *gyrA rpoB ara*. JM101 is F′ $traD_{36}$ *proAB* $lacI^qZ\Delta15/\Delta(lac\ pro)$ *supE thi*. *E. coli* strain i^-p^+ is F′ *lac pro*/$\Delta(lac\ pro)_{xIII}$ *thi*. All were obtained from J. Normanly. XACΔTK is $F^-/\Delta(lac\ pro)_{xIII}$ *ara gyrA rpoB* $argE_{am}$ *nal thi* Δ*thyA recA*::*Tn10* and was obtained from Choll W. Kim (UCLA). Plasmids pGFIB-1 and pDaYQ are described in Normanly et al. (1986b and 1990, respectively). Plasmid pThyA is pACYC184 with $ThyA_{amber166}$ cloned into the *Hind*III site and was the gift of Choll W. Kim (UCLA). Plasmid pCRS containing the cysteinyl-tRNA synthetase was the gift of G. Eriani and J. Gangloff (CNRS, Strasbourg, France).

Design and Cloning of tRNA Genes. Genes for tRNAs were constructed from six oligonucleotides and cloned into the *Eco*RI and *Pst*I sites in pGFIB-1 if the tRNA was to be used *in vivo* or in pUC18 if the tRNA was to be used solely *in vitro*. The exceptions to this are Cys73A and CysGCA, which were cloned into pGFIB-1, and CysGAA, which was cloned into pUC19. Each tRNA gene included a T7 RNA polymerase promoter, the tRNA gene, and a downstream *Bst*NI site (Figure 1B). Cleavage with *Bst*NI produces a transcription template which will produce a tRNA with the proper 3′ CCA terminus. Annealing and ligation of the oligonucleotides and cloning were carried out according to the methods of Normanly et al. (1986a). All tRNAs were sequenced using by the dideoxy method of Sanger et al. (1977).

In Vivo Genetic Methods of Studying Identity. Suppression efficiency was measured by comparing β-galactosidase levels produced by the suppressor tRNA and an episomal *lacI*–$lacZ_{amber}$ fusion in strain XAC-1 with a reference, *E. coli* strain i^-p^+. β-Galactosidase activity was determined using the method of Miller (1972). The cysteine-specific amber suppression assay (Michaels et al., 1990; C. W. Kim and J. Miller, personal communication) utilized a cysteine requiring amber mutation in the thymidylate synthetase gene (Dev et al., 1988). The suppressor tRNA of interest and pThyA (with the $thyA_{amber}$ gene) were transformed into strain XACΔTK (Thy–) and assayed for growth on minimal M9 medium (Miller, 1972) which lacked thymidine.

DHFR Assays. DHFR expression and purification was performed using a variation of the method described by Normanly et al. (1986a). DHFR was purified from XAC-1 cells using a compatible plasmid system. The two plasmids were pGFIB-1::suppressor-tRNA and pDaYQ (which contained $fol_{amber10}$, the mutant DHFR gene). Cells were grown to an optical density (λ = 600 nm) of 0.6 and then induced with 1 mM IPTG and grown for another 8 h. After recovery, cells were resuspended in 40 mM Tris pH 8.0 and then frozen in liquid nitrogen. Lysis was by lysozome-Brij58 treatment, after which the extract was treated with DNAseI. A 50% ammonium sulfate cut was performed on the extract, followed by a 100% cut on the supernate. The pellet was resuspended in dialysis buffer (50 mM potassium phosphate pH 6.0, 200 mM KCl, 500 μM EDTA, 1 mM DTT), mixed with 3 mL of methotrexate sepharose (Pierce), and dialyzed overnight. The methotrexate sepharose was then batch-washed or washed in column with 200 mM potassium phosphate pH 6.0, 1 M KCl, 1 mM DTT. The DHFR was eluted in a buffer containing folic acid (200 mM potassium borate pH 9.0, 1 M KCl, 1 mM DTT, 1 mM EDTA, 2 mM folic acid). Fractions containing DHFR were combined and dialyzed against KP8 buffer (50 mM potassium phosphate pH 8.0, 1 mM DTT). The DHFR was purified away from nucleic acids, other proteins, and folic acid by MonoQ FPLC using a linear gradient from 0 to 400 mM KCl with a slope of 16 mM KCl/min at a flow rate of 1 mL/min. Fractions containing DHFR were pooled, dialyzed against 1 mM DTT, and then concentrated with a Centricon10 filter unit (Amicon). The protein was derivatized and sequenced at the Caltech Biopolymer Synthesis & Analysis Resource Center, Protein/Peptide Sequencing Laboratory. To more accurately quantitate cysteine, the DHFR was first pyridylethylated and purified on reverse-phase C18 HPLC (D. Teplow and T. Bauer, personal communication) before sequencing.

Purification of the Cysteinyl-tRNA Synthetase (CysRS). Cysteinyl-tRNA synthetase was purified from an overproducing strain of JM101 which carried plasmid pCRS (Eriani et al., 1991) using a variation on the method of Hou et al. (1991). Two liters of cells were grown to an absorbance (λ = 600 nm) of 2 in LB medium supplemented with 100 μg/mL of ampicillin, harvested, and resuspended in 20 mL of lysis buffer (50 mM Tris pH 7.6, 1 mM EDTA, 1 mM DTT, 500 μM PMSF). The cells were washed twice in lysis buffer,

sonicated, and centrifuged at 100000g (40 000 RPM in a Beckman 70.1 Ti rotor) for 1 h at 4 °C. This S100 extract was loaded onto a Q-sepharose column which had been equilibrated with 50 mM Tris pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 500 μM PMSF and washed with 158 mL of this buffer. CysRS was eluted with a linear gradient of 0–500 mM KCl with a slope of 6.7 mM KCl/min and a flow rate of 2 mL/min. Fifty 3-mL fractions were collected; six active fractions were pooled and dialyzed (buffer: 50 mM Tris pH 7.6, 10 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 500 μM PMSF) and concentrated with a Centricon10 filter unit (Amicon). Glycerol was added to a final concentration of 50%, and the stock was stored at –20 °C. The final protein concentration was 13 μg/μL, and the activity was 680 units/mg protein (unit definition: 1 nmol of aminoacylated native $tRNA^{Cys}$ per min). The synthetase was approximately 25–50% pure and was judged free of RNAse activity by incubating it under aminoacylation conditions (see below, but with 15 mM $MgCl_2$ rather than the usual 7 mM) with 5 μM native $tRNA^{Cys}$ (Subriden RNA) for 1 h at 37 °C and then checking for degradation products on a 10% polyacrylamide (29:1 cross-link ratio), 8 M urea, 1× TBE gel.

In Vitro Transcription of tRNAs. Plasmid DNA was prepared using CsCl gradients or by anion exchange chromatography (with Qiagen (plasmid) tip 500 columns). Plasmids were then digested with restriction endonuclease *Bst*NI, extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform, precipitated, and recovered in TE8 (10 mM Tris pH 8.0, 1 mM EDTA). Transcriptions used the following conditions: 0.1 μg/μL digested DNA, 40 mM Tris pH 8.3, 20 mM $MgCl_2$, 1 mM spermidine, 5 mM DTT, 5 mM NTPs (Pharmacia), 10 mM GMP (Pharmacia), 50 μg/mL acetylated BSA (New England Biolabs), 7 units/mL pyrophosphatase (Sigma), 0.8 units/μL RNAsin (preincubated 20 minutes on ice with 20 mM DTT), and 26 units/μL T7 RNA polymerase (prepared by M. Saks and J. Sampson). Reactions were incubated for 4–5 h at 37 °C. Transcription reactions were terminated by adding 0.05 volume 0.5 M EDTA and 0.1 volume 3 M NaOAc and were then extracted with phenol equilibrated with 300 mM NaOAc pH 5.3 and precipitated.

The products were recovered in TE7.5 (10 mM Tris pH 7.5, 1 mM EDTA) and mixed with equal volumes of RNA loading buffer heated to 85–90 °C for 3 mins and then loaded onto 40-cm-long denaturing 8% polyacrylamide (19:1 cross-link ratio) gels (except for AS1 and AS2, which were purified on 10% polyacrylamide, 29:1 cross-link gels). Gels were run at 600–900V. RNA was visualized by UV shadowing, excised from the gel, and eluted twice with 200 mM KOAc pH 5.4, 10 mM EDTA at 4 °C. The RNA was precipitated and recovered in TE7.5 (10 mM Tris pH 7.5, 1 mM EDTA) three times. A fourth precipitation was carried out using 2 M NH_4-OAc in place of 0.3 M NaOAc. The RNA was then dissolved in 10 mM HEPES pH 7.4 and stored at –20 °C. The RNA was quantitated by measuring the absorbance at 260 nm of a nuclease-digested sample (1 μL of 0.9 μg/μL RNAseA, 11 units/μL RNAse T1, and 0.2 units/μL RNAse T2 in 700 μL of diluted tRNA, incubated overnight at room temperature). Extinction coefficients were calculated from the sequence of the tRNA.

Aminoacylation Reactions. Aminoacylation reactions were performed at 37 °C in 30 mM HEPES pH 7.4, 2 mM DTT, 15 mM KCl, 7 mM $MgCl_2$, 2 mM ATP, 20 μM cold cysteine, 0.4 μCi/μL ^{35}S-cysteine (stabilized, SJ15232, Amersham, nominal specific activity approximately 1300Ci/mmol), and cysteinyl-tRNA synthetase. Synthetase was used at a final dilution of 1:200 (65 μg/mL final synthetase concentration) to 1:100 000 (0.13 μg/mL final synthetase concentration). The final protein concentration in all reactions was adjusted to 0.1 μg/μL with acetylated BSA. RNA was prepared at 2× final concentration in distilled water and was heated to 90 °C and allowed to slow cool before use. At appropriate time points, 8-μL samples were spotted onto 1-in. × 0.5-in. segments of prepared Whatman 3MM paper. These papers had been soaked 1–2 days before in a solution of TCA and amino acids (10% TCA, 10 mM DTT, 25 μM all amino acids except cysteine, 100 μM cysteine) and then allowed to dry. Spotted papers were soaked in 10% TCA and washed twice in 10% TCA, three times in 5% TCA (all 20 min per wash), and once in 95% ethanol (15 min). The washed papers were dried, placed in vials with 4 mL of fluor (15.3 g of PPO in 4 L of Mallinkrodt ScintillAR toluene), and counted with a Beckman LS7800 scintillation counter (window 0–1000). Several control reactions were performed without tRNA in order to estimate background counts.

Data Analysis. Backgrounds cpms (determined from reactions which contained no RNA) were subtracted from the counting data and aminoacylation rates calculated by linear regression. Enzfitter (Biosoft), a non-linear regression program designed for the calculation of kinetic parameters, was used to generate Michaelis–Menten plots and Eadie–Hofstee plots, as well as the values of k_{cat}, K_m, and k_{cat}/K_m of aminoacylation by the cysteinyl-tRNA synthetase. ^{35}S-Cysteine has a high rate of nonradiolytic cleavage, making exact determinations of specific activities difficult. Values for k_{cat} were therefore converted from cpm min^{-1} to nmol min^{-1} mg protein^{-1} in one of the two following ways. (1) Two aminoacylation reactions with 1 μM native $tRNA^{Cys}$ were allowed to charge to completion using either ^{14}C or ^{35}S labeled cysteine. Since ^{14}C cysteine does not suffer from nonradiolytic degradation, this reaction could be used to verify that the ^{35}S-cysteine reaction had completely aminoacylated the tRNA. The ^{35}S reaction could then be used to develop a conversion factor for cpm to nM. The data in Table III were converted in this fashion. (2) Using data from two separate reactions of the type described above, a standard value for k_{cat} of a T7-transcribed $tRNA^{Cys}$ was determined (in nmol min^{-1} mg protein^{-1}); a conversion factor could then be determined from the experimentally determined value of k_{cat} (in cpm min^{-1} mg protein^{-1}) for a T7 transcript of $tRNA^{Cys}$. The data in Tables I, II, and IV were calculated in this way.

RESULTS

Construction and in Vivo Studies of Amber Suppressor Mutants of $tRNA^{Cys}$. To begin a dual study of cysteine identity and recognition, a cysteine amber suppressor tRNA (Cys-CUA) was constructed in plasmid pGFIB-1. Such a gene had been constructed before (Normanly et al., 1986b), but it did not contain a T7 RNA polymerase promoter and its identity had been assayed using β-galactosidase (rather than DHFR) as the reporter gene. The suppression efficiency for the CysCUA amber suppressor tRNA was 11–24%, which is consistent with the results (17–50%) of Normanly et al. (1986b). The ability of tRNA CysCUA to suppress a mutant with an amber codon in the cysteine requiring active site of thymidylate synthetase indicates that it is aminoacylated at some level by cysteine, although this assay does not eliminate the possibility that other synthetases are also aminoacylating the tRNA. To determine the identity of CysCUA, a second plasmid, pDaYQ, which contains the $fol_{amber10}$ gene under

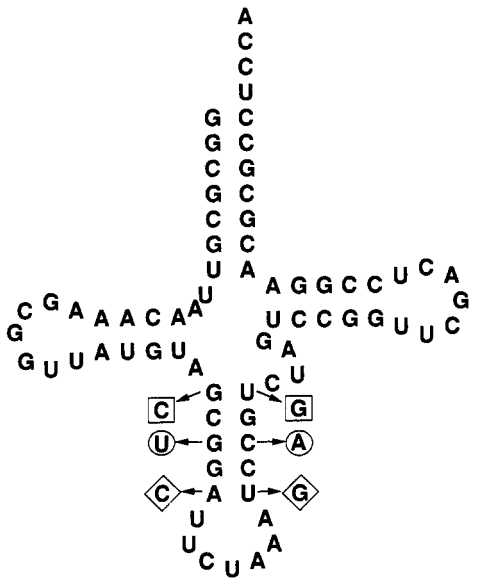

FIGURE 2: RNA sequence of T7-transcribed amber suppressor mutant tRNAs. The background sequence is the cysteine amber suppressor tRNA. The marked changes occur in the following mutant tRNAs: all changes, acm2; ◇, acm5; ○, acm7; □, acm8.

the control of a *tac* promoter, was transformed into the strain carrying pGFIB:CysCUA, and DHFR protein was purified and sequenced. The relative ability of the various synthetases to aminoacylate the tRNA is approximately reflected by the amino acid composition of the protein at position 10; for CysCUA the results were 94% Cys, 6% Gln (Table I). These results independently confirm that the CysCUA retains its cysteine identity *in vivo*.

Since the $tRNA^{Cys}$ amber suppressor retained its identity, an attempt was made to define the sequence elements which would direct aminoacylation by the cysteinyl-tRNA synthetase *in vivo*. To do this, several tRNAs were constructed which had differing amounts of the $tRNA^{Cys}$ sequence modified to the sequence of an alanine (UGC) tRNA. If a region of the tRNA contained important identity elements then mutations in that region should alter cysteine identity *in vivo*. Figure 2 shows acm2, an amber suppressor tRNA derived from CysCUA in which three base pairs in the anticodon stem have been mutated so that the stem is identical to the $tRNA^{Ala}$ (UGC) sequence. Its suppression efficiency is 5–12%, and it suppresses the cysteine-specific thymidylate synthetase amber mutation (Table I). However, the DHFR assay indicates that *in vivo* acm2 has lost much of its cysteine identity, and the tRNA is primarily aminoacylated with glutamine (Table I). To determine whether one or more of the three base pairs which had been mutated in acm2 was an identity element for the cysteinyl-tRNA synthetase, three tRNAs were constructed, each differing from the sequence of CysCUA in only one of the base pairs mutated in acm2 (acm5, acm7, and acm8, Figure 2). Table I lists their suppression efficiencies and the results of the thymidylate synthetase and DHFR assays. Surprisingly, all three tRNAs retained their *in vivo* identity fairly well, ranging from 89 to 93% cysteine in the DHFR assay; the variations between the DHFR assays for CysCUA, acm5, acm7, and acm8 are within the margin of error of the assay. There are two possible interpretations for these results: either the anticodon stem mutations are affecting recognition by the cysteinyl-tRNA synthetase, or they are affecting the competition between synthetases. *In vitro* aminoacylation experiments were done to differentiate between these possibilities.

Comparison of the in Vitro Aminoacylation Kinetics of Native and T7 Transcribed $tRNA^{Cys}$. Before progressing to a study of the aminoacylation kinetics of mutant tRNAs, it was necessary to determine if a T7 transcript of $tRNA^{Cys}$ behaved similarly to the native tRNA. Both tRNAs were aminoacylated in 30 mM HEPES pH 7.4 with 7 mM $MgCl_2$ and 2 mM ATP. A $MgCl_2$ titration curve indicates that these conditions are optimal for the T7 transcript; the native tRNA is aminoacylated better at lower $MgCl_2$ concentrations. At a concentration of 1 μM tRNA, there is a 1.5-fold increase in the rate of aminoacylation of the native tRNA at 3 mM $MgCl_2$ as compared to the rate of aminoacylation at 7 mM $MgCl_2$. The transcript, on the other hand, has a nearly 9-fold reduction in the initial rate of aminoacylation at the lower salt concentration. Under the higher salt (7 mM $MgCl_2$) conditions, both RNAs can be fully aminoacylated using substrate levels of enzyme in less than 30 s. The T7 transcript however, has a 1.6-fold increase in k_{cat} and a somewhat unusual 9.5-fold increase in K_m compared to the native tRNA. Overall, k_{cat}/K_m for the native tRNA is increased 5.5-fold compared to the T7 transcript (Table II).

In Vitro Studies of Amber Suppressor $tRNA^{Cys}$ Mutants. The wild-type amber suppressor tRNA (CysCUA) and the four mutants acm2, acm5, acm7, and acm8 were transcribed, purified, and aminoacylated. Table I shows the values for k_{cat}, K_m, and k_{cat}/K_m (the specificity constant) associated with the various mutants. Also shown is the change in specificity, the degree to which k_{cat}/K_m is reduced or increased compared to a wild-type $tRNA^{Cys}$. The range of tRNA concentrations used with the amber suppressors was 2–20 mM. Since the measured K_m in many cases was greater than 15 μM, determining the individual values of K_m or k_{cat} for many of the tRNAs required significant extrapolation. Although Enzfitter calculated a standard error for these values, another independent determination of the kinetic constants could easily produce calculated values outside of these ranges. However, the qualitative effect on the individual kinetic constants, i.e., did they decrease or increase, was clear. The value of k_{cat}/K_m, on the other hand, does not require significant extrapolation, since its value is calculated from the x-intercept of an Eadie–Hofstee (v/[tRNA] vs v) plot (i.e., from data points where [tRNA] $\ll K_m$).

Compared to a wild-type transcript (CysGCA), CysCUA is reduced 3100-fold in k_{cat}/K_m and acm2 is reduced 2400-fold, while acm5 and acm7 are reduced 2900- and 3500-fold, respectively. Mutant acm8 is reduced 1200-fold in k_{cat}/K_m relative to a wild-type transcript. All mutants had a decreased value of k_{cat} and an increased value of K_m relative to a wild-type transcript (Table I).

These values for the specificity constant, k_{cat}/K_m, are not consistent with the *in vivo* identity data. Even if one considers an error of a factor of 2 in the calculation of k_{cat}/K_m, there seems to be no possible correlation beteen *in vivo* identity and the *in vitro* specificity constant under these conditions. An Eadie–Hofstee plot for tRNA acm2 (37% Cys *in vivo*) shows data points arranged in a vertical line, indicating a high K_m but preventing direct measurement of either of the individual values of k_{cat} or K_m. This high value of K_m may be the factor which causes its loss of *in vivo* identity. In any case, the large reductions in k_{cat}/K_m seen in all of the amber suppressor tRNAs suggested that the anticodon plays an important role in the recognition of $tRNA^{Cys}$ by the cysteinyl-tRNA synthetase.

Effects of Mutations in the Anticodon and Discriminator on Recognition by Cysteinyl-tRNA Synthetase. In order to

Table I: Kinetic Parameters and Results of *in Vivo* Experiments for Mutant Cysteine Amber Suppressor tRNA Molecules[a]

	k_{cat} (nmol min^{-1} mg protein^{-1})	K_m (μM)	k_{cat}/K_m	specificity change (-fold)	suppression efficiency (% wild type)	suppression of ThyA amber	DHFR (% at pos. 10) Cys	DHFR (% at pos. 10) Gln/
CysGCA[b]	1100 ± 50	3.8 ± 0.4	290	*e*	*e*	*e*	*e*	*e*
CysCUA	4.8 ± 0.5	51 ± 7	0.093	−3100	11–24	(+)	94	6
acm2	*c*	*c*	0.12	−2400	5–12	(+)	37	63
acm5	3.8 ± 0.5	37 ± 6	0.10	−2900	4	(+)	90	10
acm7	2.3 ± 0.4	28 ± 7	0.082	−3500	9	(+)	93	5
acm8[d]	5.6 ± 0.6	20 ± 3	0.28	−1200	20	(+)	89	11

[a] Specificity change indicates the level of increase or decrease in k_{cat}/K_m relative to that of a T7-transcribed tRNA^{Cys}. See text for details about tRNAs. [b] These data from Table II are reprinted for convenience. [c] The Eadie–Hofstee plot for this tRNA shows points in a vertical line, indicating a high K_m but preventing the direct measurement of k_{cat} or K_m. [d] These data are from a separate experiment; specificity change here is derived from a separate calculation of k_{cat}/K_m for a wild-type transcript (CysGCA). [e] Not applicable. [f] Includes Glu.

Table II: Kinetic Parameters for Native and T7-Transcribed *E. coli* Cysteine tRNAs[a]

tRNA	k_{cat} (nmol min^{-1} mg protein^{-1})	K_m (μM)	k_{cat}/K_m	specificity change (-fold)
native tRNA$^{Cys}_{GCA}$	680 ± 60	0.4 ± 0.1	1600	+5.5
T7 tRNA$^{Cys}_{GCA}$	1100 ± 50	3.8 ± 0.4	290	*b*

[a] Specificity change indicates the level of decrease or increase in k_{cat}/K_m relative to T7-transcribed tRNA$^{Cys}_{GCA}$. [b] Not applicable.

Table III: Kinetic Parameters for Anticodon and Discriminator Mutants[a]

	codon recognized	k_{cat} (nmol min^{-1} mg protein^{-1})	K_m (μM)	k_{cat}/K_m	specificity change (-fold)
CysGCA	Cys	1100 ± 40	3.1 ± 0.4	350	*c*
CysACA	Cys	7.3 ± 0.6	38 ± 5	0.19	−1800
CysUCA	opal	4.6 ± 0.2	25 ± 2	0.18	−1900
CysCCA	Trp	4.1 ± 0.4	30 ± 5	0.14	−2500
CysGAA	Phe	950 ± 40	3.3 ± 0.4	290	−1.2
CysGUA	Tyr	29 ± 2	28 ± 2	1.1	−320
CysGGA	Ser	71 ± 7	25 ± 4	2.8	−120
CysGCU	Ser	30 ± 5	10 ± 3	2.9	−120
CysGCG	Arg	45 ± 2	18 ± 2	2.5	−140
CysGCC	Gly	141 ± 6	16 ± 1	8.6	−41
Cys73A	Cys	0.11 ± 0.02	4.0 ± 1.6	0.027	−13 000
Cys73G	Cys	*b*	*b*	*b*	*b*
Cys73C	Cys	0.76 ± 0.07	7.1 ± 1.5	0.11	−3200

[a] Specificity change is the level of decrease or increase in k_{cat}/K_m relative to a T7-transcribed tRNA$^{Cys}_{GCA}$. See text for explanation of mutants. [b] No aminoacylation detected. [c] Not applicable.

determine the role of the anticodon nucleotides in recognition by the cysteinyl-tRNA synthetase, all three single base changes were made at each of the anticodon positions. The mutants are labeled CysXXX, where XXX indicates the anticodon present in the tRNA. The importance of the discriminator nucleotide in other systems (and the fact that only one other isoaccepting group in *E. coli*, glycine, has the U73 discriminator (Komine et al., 1990; Sprinzl et al., 1991)) induced us to construct the three single base change mutants at this position (these mutants are labeled Cys73X, where X is the nucleotide in the discriminator position). After transcription and gel purification, the kinetic parameters for these tRNAs were determined.

Table III shows the experimentally determined values of k_{cat}, K_m, and k_{cat}/K_m for the mutants, as well as the degree to which k_{cat}/K_m was reduced relative to a wild-type tRNA^{Cys} transcript (specificity change). As with the amber suppressor tRNAs, the range of tRNA concentrations (1–20 μM) does not allow the most accurate determination of individual values of k_{cat} or K_m; nevertheless, the qualitative effect of the mutation on the kinetic parameters is usually quite clear.

Mutations in nucleotide 34, the wobble position, clearly had dramatic effects on the aminoacylation kinetics. All three replacements caused reductions in k_{cat}/K_m of 3 orders of magnitude relative to a wild-type transcript. All three substitutions increased K_m and decreased k_{cat}. Nucleotide substitutions at position 35 had highly variable effects. Interestingly, the C35A (CysGAA) mutation had essentially no effect on either K_m or k_{cat}. The C35U (CysGUA) and C35G (CysGGA) mutations, on the other hand, caused reductions in k_{cat}/K_m of 2 orders of magnitude. Both seem to cause increases in K_m and substantial reductions in k_{cat}. Less dramatic effects on aminoacylation occur when nucleotide 36 is replaced. The three substitutions produce reductions in k_{cat}/K_m of 2 (A36U (CysGCU) and A36G (CysGCG)) or one (A36C (CysGCC)) order(s) of magnitude. All of these substitutions cause reductions in k_{cat} and increases in K_m compared to a wild-type transcript.

The most dramatic effects caused by the replacement of a single nucleotide are seen with the substitution of the discriminator nucleotide (position 73). The U73C mutation (Cys73C) has the least effect, causing the total catalytic efficiency to decrease by 3200-fold compared to a wild-type transcript. Although the poor aminoacylation of this substrate prevents accurate determination of individual kinetic parameters, the data suggest that the effect of the U73C mutation is primarily caused by a reduction in k_{cat}, with a minimal increase in K_m. The U73A (Cys73A) mutant has a 13 000-fold reduction in k_{cat}/K_m, again with a suggestion that the effect is almost entirely caused by a decrease in k_{cat}, with a minor effect on K_m. Mutant Cys73G (U73G) could not be aminoacylated to any significant degree, and so it presumably had an even more dramatic reduction in k_{cat}/K_m than Cys73A.

Localization of Other Recognition Elements. To help further define the recognition elements of the cysteine tRNA, a set of tRNAs were designed in which structural domains of the cysteine tRNA were replaced by the equivalent regions from a heterologous tRNA. The domains (shown in Figure 3A) are the acceptor stem, the anticodon stem and loop, and the tertiary domain (comprising the D stem and loop, TΨC stem and loop, extra arm, and propeller twist nucleotides). Dividing the tRNA into these three domains should not affect the interactions that maintain the three-dimensional shape of the tRNA, which occur between nucleotides in the tertiary domain. Hybrid tRNAs should therefore adopt the three-dimensional configuration of the tRNA species which contributed the tertiary domain.

As an initial experiment, two tRNAs, AS1 and AS2 (Figure 3B,C; M. Saks and J. Sampson, personal communication), which are based on a D4V5 alanine sequence but with the cysteine anticodon and a C73 discriminator, were tested for aminoacylation by the cysteinyl-tRNA synthetase. These tRNAs differ from tRNA^{Cys} in both a sequence-specific and

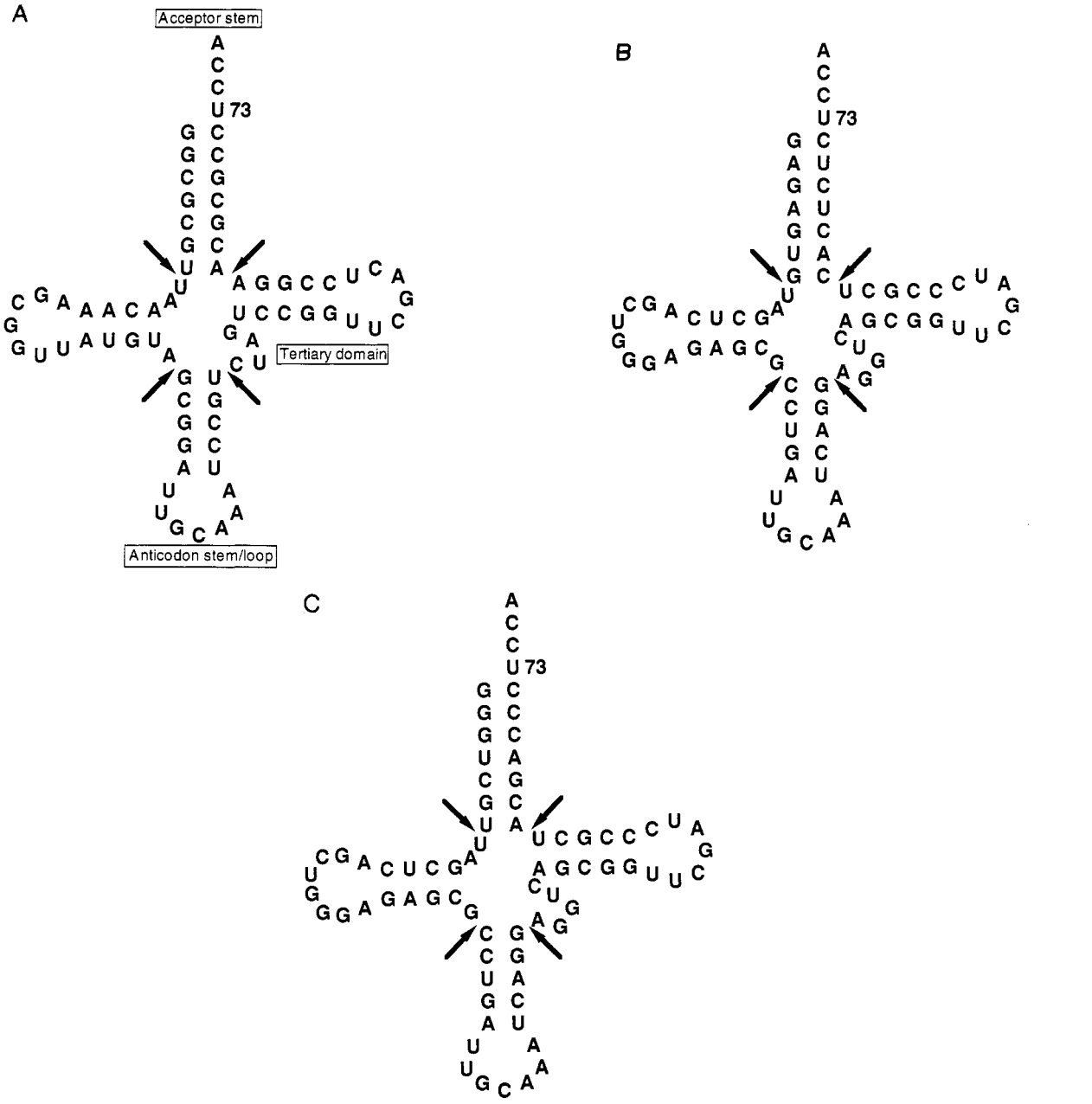

FIGURE 3: RNA sequence of T7-transcribed (A) $tRNA^{Cys}$, (B) AS1, and (C) AS2, showing the breakpoints for the three structural regions used in the domain replacement experiments.

a structure-specific fashion. $tRNA^{Cys}$ is a D3V4 tRNA; AS1 and AS2 as indicated are type D4V5. Crystallographic evidence from yeast $tRNA^{Asp}$ [see Westhof et al. (1985)] suggests that D3V4 tRNAs adopt a somewhat different three-dimensional conformation than D4V5 tRNAs. In addition, the number and location of the dihydrouridine residues (which are present as unmodified, stackable U residues in the transcripts) which define the D loop and the unusual G15: G48 Levitt pair found in $tRNA^{Cys}$ vary between $tRNA^{Cys}$ and the AS tRNAs. Finally, the acceptor stems of the two tRNAs are different from that of $tRNA^{Cys}$ at six of seven base pairs in AS1 and two of seven base pairs in AS2. Thus, if the anticodon and discriminator are the only major recognition elements, these tRNAs should be aminoacylated about as well as the U73C $tRNA^{Cys}$. Since neither tRNA was aminoacylated to any significant extent using substrate levels of enzyme, it suggests that there are other recognition elements present in the cysteine tRNA.

The five mutant tRNAs which were constructed are shown schematically in Figure 4. AS1 was used as the source for heterologous sequences. Three of the tRNAs are single substitutions of the domains. Two additional tRNAs with substitutions in two of the domains were constructed to investigate whether any recognition elements in the regions acted independently. The tRNAs are designated by the Greek letter Δ followed by the name of the domains which have been replaced with AS1 sequences (*acc*eptor stem, *anti*codon stem/loop, or *tertiary*). All of the tRNAs had anticodon GCA and the U73 discriminator to facilitate detection of other recognition elements for CysRS. Table IV lists the five tRNAs with their measured kinetic constants, K_m, k_{cat}, and k_{cat}/K_m.

The single domain replacement hybrids varied widely in cysteine acceptor activity. Replacement of the cysteine anticodon stem (mutant Δanti) with the equivalent domain from the AS1 tRNA causes a decrease in K_m with a concomitant reduction in k_{cat}. This results in a mild 1.7-fold increase in k_{cat}/K_m for aminoacylation as compared to a wild-type $tRNA^{Cys}$ transcript (CysGCA). Replacement of the tertiary domain with AS1 sequences (mutant Δtertiary) results in an increase in K_m and a decrease in k_{cat}. The value of

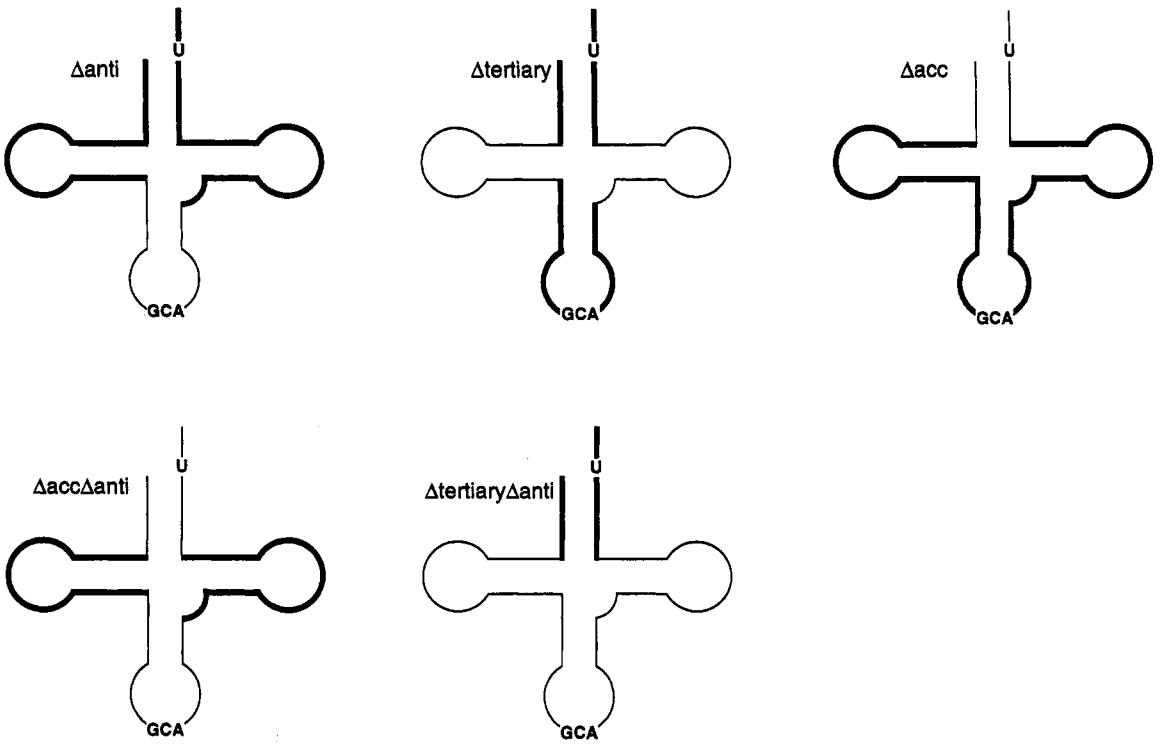

FIGURE 4: Schematic diagram of the domain replacement mutants. Thick lines represent cysteine sequence, thin lines AS1 sequence.

Table IV: Kinetic Parameters for Domain Replacement Mutants of *E. coli* Cysteine tRNA[a]

	k_{cat} (nmol min^{-1} mg protein^{-1})	K_m (μM)	k_{cat}/K_m	specificity change (-fold)
CysGCA[b]	1100 ± 50	3.8 ± 0.4	290	*c*
Δanti	680 ± 30	1.4 ± 0.2	480	+1.7
Δtertiary	230 ± 30	26 ± 5	8.7	-33
Δacc	1200 ± 30	10.3 ± 0.5	120	-2.4
ΔaccΔanti	850 ± 20	3.0 ± 0.2	290	1.0
ΔtertiaryΔanti	65 ± 5	18 ± 2	3.6	-81

[a] Specificity change indicates the level of increase or decrease in k_{cat}/K_m relative to a T7-transcribed $tRNA^{Cys}_{GCA}$. See text for details about tRNAs. [b] Data from Table II repeated here for convenience. [c] Not applicable.

k_{cat}/K_m is thus reduced 33-fold in this mutant from that of a CysGCA transcript. Replacement of the acceptor stem with the AS1-U73 stem in mutant Δacc causes an increase in K_m and an insignificant increase in k_{cat}. overall, k_{cat}/K_m is reduced a minor 2.4-fold compared to a wild-type cysteine transcript.

Double replacement mutant ΔaccΔanti has decreased values of K_m and k_{cat} when compared to the single mutant Δacc, the same qualitative effects caused by the single mutant Δanti with regard to the wild-type $tRNA^{Cys}$. The value of k_{cat}/K_m is essentially the same as the wild-type transcript, the result which would be expected from two mutations which cause respectively an increase of 1.7-fold and a decrease of 2.4-fold in k_{cat}/K_m with regard to CysGCA [two mutations acting independently would be expected to cause an increase or decrease in k_{cat}/K_m approximately equal to the product of their individual effects; see Sampson et al. (1992)]. The two replacements in ΔtertiaryΔanti cause a reduction in the values of k_{cat} and K_m compared to single mutant Δtertiary, consistent with the effects of the anticodon stem replacement on a wild-type transcript. However, the overall effect on k_{cat}/K_m (an 81-fold reduction) caused by the mutations in ΔtertiaryΔanti is not what would be predicted (an approximately 20-fold reduction) from two mutations which individually cause a 1.7-fold increase and a 33-fold reduction in k_{cat}/K_m.

DISCUSSION

Discrimination of tRNAs by synthetases *in vivo* is dictated by a combination of two factors: the total catalytic efficiency of the reaction with the cognate synthetase and the catalytic efficiency of the reaction with the 19 incorrect synthetases. Competition between all of the synthetases found within the cell ultimately defines the amino acid acceptance of a tRNA *in vivo* [see Sherman et al. (1992) and Swanson et al. (1988)]. The elements which define the amino acid acceptance of a tRNA *in vivo* are called the identity set, and the overall process is tRNA identity. The elements which each of the 20 synthetases use to define cognate tRNAs are called recognition sets, and the process is tRNA recognition. Recognition elements can act by possessing functional groups which interact with synthetase or by providing a structure which puts another element in the proper orientation to interact with the synthetase. All recognition elements by definition are positive, that is, the presence of the element aids the creation of a productive interaction with that synthetase. Identity elements can be either positive or negative. A positive identity element is a recognition element, assisting a productive interaction with the cognate synthetase. A negative element on the other hand, helps prevent productive interactions with noncognate synthetases. Negative elements are critical in the cell, since many synthetases use some of the same tRNA nucleotide elements to help recognize cognate tRNAs [see Schulman (1991) for review].

The results of *in vivo* and *in vitro* experiments provide information on different aspects of the tRNA/synthetase interaction. Since *in vivo* experiments occur in a milieu where all 20 synthetases are present, they provide information on the nature of the identity set. They cannot, however, distinguish whether a change in amino acid acceptance is caused by removing an identity element for a synthetase or adding an identity element for another. Furthermore, the

necessity of working with amber or opal suppressors prevents direct experimentation on the effects of the anticodon nucleotides [although an alternate method which uses an initiation assay can study anticodon effects, at least within the context of a $tRNA^{fmet}$ body, see Chattapadhyay et al. (1990)]. *In vitro* experiments can only examine the recognition set, since the element of competition between synthetases has been removed. The weakness of these experiments is that they cannot detect negative identity elements. The two types of experiments complement each other so that together it is possible to obtain a more detailed view of the process of tRNA: synthetase interactions.

It is clear from the *in vitro* experiments that the cysteinyl-tRNA synthetase uses a variety of widely spaced elements as part of its recognition set. These elements include the anticodon nucleotides, the discriminator nucleotide, a feature within the tertiary domain of the tRNA, and possibly a modified nucleotide or nucleotides (or a structural feature produced by a modified nucleotide). The results also suggest that the acceptor stem and anticodon stem play little or no role in recognition. Since it was not possible to make all substitutions at all positions in the tRNA, the possibility exists that there are undiscovered recognition elements within one of the domains. The probability of unknown recognition elements remaining in the acceptor stem seems slight, however, as only one base pair in this structure (G1:C72) was not changed in tRNA Δacc, and this base pair is found in 15 isoaccepting groups in *E. coli*.

The results of the aminoacylation reactions with native $tRNA^{Cys}$ suggest the possibility that one or more of the seven modified nucleotides (see Figure 1) may be a recognition element. Since the thymidine and pseudouridine in the TΨC loop are found in all tRNAs, it seems unlikely that these residues are involved. Any of these nucleotides could influence recognition by either possessing functional groups which interact directly with the synthetase or maintaining important structural features of the tRNA. The dihydrouridine at position 21 might have the latter effect. In yeast $tRNA^{Phe}$ and $tRNA^{Asp}$, this residue is coplanar with the 8:14 tertiary interaction and stacked on nucleotide 46. Since dihydrouridine is not planar, this residue cannot interact in such a fashion in native $tRNA^{Cys}$, although the U residues found in the T7 transcript might. Since the native and T7-transcribed tRNAs are aminoacylated best at different $MgCl_2$ levels (data not shown), there is reason to suspect that this latter explanation is the most likely.

The domain replacement experiments suggest that there are no recognition elements in the acceptor stem or anticodon stem. The minor effects on k_{cat}/K_m caused by these mutants are most likely caused by reorienting other important elements (perhaps the discriminator, CCA terminus, or the anticodon) or by making small changes in helix geometry or some other element of the tertiary structure of the molecule. It does appear that there are recognition elements and/or important structural features within the tertiary domain of $tRNA^{Cys}$. This is particularly interesting since the body of *E. coli* $tRNA^{Cys}$ may have a conformation which is different from that of yeast $tRNA^{Phe}$ or $tRNA^{Asp}$ (Kim et al., 1974; Westhof et al., 1985). *E. coli* $tRNA^{Cys}$ has an unusual G15:G48 Levitt pair, found in no other tRNA from this species and unique to *E. coli* among other sequenced cysteine tRNAs. Sampson et al. (1990) found that replacing the G15:C48 Levitt pair in yeast $tRNA^{Phe}$ with the other common pair A15:U48 resulted in a small, 1.6-fold decrease in k_{cat}/K_m, while replacement with G15:G48 caused a 4-fold reduction in k_{cat}/K_m. There was an associated reduction in the rate of lead-catalyzed tRNA cleavage, a process which is highly dependent upon having a yeast phenylalanine tRNA-like structure (Behlen et al., 1990). Furthermore, evidence from crystallographic (Westhof et al., 1985) and functional studies of yeast $tRNA^{Asp}$ (Giegé et al., 1990; Perret et al., 1992) suggest that D3V4 tRNAs such as *E. coli* $tRNA^{Cys}$ and yeast $tRNA^{Asp}$ are structurally distinct from D4V5 molecules like $tRNA^{Phe}$.

The element which contributed the most to cysteine recognition *in vitro* was the discriminator nucleotide U73. The mildest mutational effect was seen in Cys73C, a pyrimidine/pyrimidine swap. Replacement of the discriminator with purines caused even more dramatic effects: Cys73A was reduced another 4-fold in k_{cat}/K_m compared to Cys73C, and Cys73G was not significantly aminoacylated under these conditions. Curiously, neither the U73A or U73C mutations seemed to cause large changes in the value of K_m; the bulk of the effect appears to be on k_{cat} (this interpretation must be viewed with some caution due to the poor aminoacylation of these substrates). This is consistent with models by Hou and Schimmel (1989a) which postulate that most discrimination *in vivo* is based on difference in k_{cat}. In the absence of a crystal structure it is impossible to determine the exact role of U73, but there are two obvious possibilities. One is that there is direct recognition of the single-stranded pyrimidine. The other possibility is that it plays a role similar to that of the G73 in *E. coli* glutamine tRNA, which participates in an intramolecular hydrogen bond which orients the CCA terminus (Rould et al., 1989). Mutations of the discriminator in $tRNA^{Gln}$ (Jahn et al., 1991) cause reductions in k_{cat}/K_m of aminoacylation by the GLnRS of 2- (G73A) to 1600- (G73U) fold. The G73A mutation in $tRNA^{Gln}$ actually decreases both K_m and k_{cat} for glutaminylation, while G73U and G73C decrease k_{cat} and increase K_m.

The anticodon nucleotides also have relatively large effects on aminoacylation kinetics, consistent with the observations that these residues play important roles in recognition by most aminoacyl-tRNA synthetases in *E. coli* (Schulman, 1991; Shimizu et al., 1992). Mutations at nucleotide 34 have the most dramatic effects, causing reductions in k_{cat}/K_m of 3 orders of magnitude, with reductions in k_{cat} and increases in K_m. Mutations in the other nucleotides cause less dramatic effects, although they all appear to be important for recognition. Interestingly, the C35A mutation, which produces a phenylalanine anticodon, has essentially the same values of k_{cat} and K_m (see below). It is ironic that the wobble nucleotide appears to be the most important for recognition by CysRS, since in codons this position can often be changed without modifying the translated protein sequence. It is also important to note that U34 is generally modified in *E. coli* tRNAs [for review see Björk et al. (1987)]; extrapolating the effect of the U34 mutation *in vivo* may therefore be difficult.

These experiments are consistent with the observations of Shimizu et al. (1992), who found that a valine tRNA with the GCA anticodon and U73 discriminator of $tRNA^{Cys}$ could be aminoacylated with cysteine *in vitro* to a plateau of approximately 300 pmol/A_{260} (compared to a plateau of approximately 1500 pmol/A_{260} for native $tRNA^{Cys}$), while mutants with A73, G73, or C73 were not aminoacylated. It is also interesting to compare and contrast our *in vitro* results with the *in vivo* results of Pallanck et al. (1992). They studied $tRNA^{Cys}$ recognition using an *in vivo* initiation system (Chattapadhyay et al., 1990) in which the anticodon of interest is incorporated into $tRNA^{fmet}$. DHFR is then prepared from a strain in which the initiation codon for the *fol* gene has been

mutated to match the anticodon on the $tRNA^{fmet}$. They found that the U73 discriminator and all three anticodon nucleotides were important for maintaining *in vivo* identity, consistent with these results. They did not test a $tRNA^{fmet}$U73 with the GAA anticodon (which our experiments predict should be cysteinylated as efficiently as $tRNA^{fmet}$ (GCA)), but previous experiments (Chattapadhyay et al., 1990) tested $tRNA^{fmet}$-A73 (GAA). They found a mixture of Phe, Met, and Val, but no cysteine. Since the protein was not derivatized prior to sequencing, it is possible that some small amount of cysteine was present and not detected.

It is interesting that mutants CysGAA and CysGCC with anticodons from $tRNA^{Phe}$ and $tRNA^{Gly2}$ are aminoacylated as well as (CysGAA) or only 41-fold less efficiently (CysGCC) than a T7 transcript corresponding to $tRNA^{Cys}$. This raises the question of which elements on $tRNA^{Phe}$ and $tRNA^{Gly2}$ are preventing misacylation by CysRS. The obvious explanation for the absence of mischarging of $tRNA^{Phe}$ is the A73 discriminator of $tRNA^{Phe}$. By attempting to aminoacylate wild-type (A73) and an A73U mutant of $tRNA^{Phe}$ with cysteine, it should be possible to determine if the discriminator is the primary defense against misacylation by the cysteinyl-tRNA synthetase or if other elements in $tRNA^{Phe}$ are involved in preventing productive interactions with CysRS. All glycine tRNAs have the discriminator U73, so some other element must be preventing misacylation with cysteine. Of the three glycine tRNAs, $tRNA^{Gly1}$ is type D3V4 and has the anticodon UCC (which would be expected to reduce k_{cat}/K_m for aminoacylation with cysteine by 3 orders of magnitude), and $tRNA^{Gly2}$ is type D4V5 and has the anticodon GCC. It is intriguing to speculate that the combination of the UCC anticodon with the D3V4 tRNA and the GCC anticodon with the D4V5 tRNA is designed to prevent misacylation by cysteine.

The *in vitro* experiments indicate that not all of the mutations act independently. tRNAs that contain two mutations that act independently may affect aminoacylation efficiency (i.e., relative k_{cat}/K_m) in proportion to the product of their effects when present alone (Sampson et al., 1992). However, the specificity constant for the cysteine amber suppressor tRNA suggests that anticodon mutations do not act independently. The reduction in k_{cat}/K_m with regard to a wild-type trancript for the amber suppressor is 3100-fold, much less than the 5–6 orders of magnitude which would be predicted for a double mutant in which the individual mutations cause reductions of 3 and 2 orders of magnitude. The Δtertiary and Δanti mutations also did not appear to act independently; in a double replacement mutant they caused a reduction in k_{cat}/K_m of 81-fold, rather than the approximately 20-fold reduction predicted from their individual effects. On the other hand, mutations Δacc and Δanti appear to act independently: the double mutant has a predicted specificity 1.4-fold less than a wild-type transcript, when the observed value is 1.0.

The experiments also underscore the fundamental observation that *in vivo* and *in vitro* experiments study different aspects of the tRNA/synthetase interaction. Surprisingly, amber suppressor tRNA acm2, which retains only 37% of its cysteine identity *in vivo*, was found to be an equivalent substrate (in terms of k_{cat}/K_m) than other amber suppressor tRNAs which retain their identity *in vivo* (89–93% cysteine). One possible explanation for these anomalous results is that the cumulative effects of the three base pair changes in acm2 improve recognition for the glutaminyl-tRNA synthetase (perhaps by repositioning the anticodon, which contains two recognition elements for GlnRS, C34, and U35 (Jahn et al., 1991)). A marginally improved affinity of acm2 for the glutaminyl-tRNA synthetase could allow the GlnRS to successfully compete with the cysteinyl-tRNA synthetase for a poor substrate. Another possibility is that the cumulative effects of the mutations allowed the glutaminyl-tRNA synthetase to compete successfully for this substrate at the concentration present *in vivo* by increasing the K_m for aminoacylation by the CysRS. These theories could be tested by conducting aminoacylation reactions with the glutaminyl-tRNA synthetase. If the latter explanation is correct, it would seem to be an exception to the rule proposed by Hou and Schimmel (1989a) that k_{cat} produces the dominant effect in determining identity. Perhaps with tRNAs that are such poor substrates, other factors, such as K_m, can be the dominant factor in determining identity *in vivo*.

It is also possible that the variations between the *in vivo* and *in vitro* results are caused by differences in the modification patterns of the tRNAs *in vivo*. This seems unlikely for several reasons. First, only six bases are different between all of the reported tRNAs which were tested *in vivo*, and only one of these (U39) is modified in $tRNA^{Cys}$ (to Ψ). While this modification is obviously absent (since U39 is absent) in acm2 (37% Cys) and should be present in CysCUA, acm7, and acm8 (89–94% Cys), it must also be absent in acm8 (90% Cys), suggesting that it is nonessential. It is also possible that one of the nucleotides which replace the six changed in acm2 could be modified, although there is no evidence among sequenced eubacterial tRNAs for modifications in any of these residues other than Ψ39 (Björk, 1986). Finally, it is possible that the mutations made in acm2 caused changes in the modification of bases outside of the anticodon stem, changes which were not present in CysCUA, acm5, acm7, or acm8. This possibility cannot be ruled out in the absence of RNA sequence information from the *in vivo* tRNAs.

Whatever the reason for this anomaly, *in vitro* aminoacylation experiments do not include the effects of competition between synthetases; hence the data only reflect the recognition set, not the complete identity set. Similarly, *in vivo* experiments study the complete identity set rather than the recognition set. Only combined studies give a true picture of the interactions of an aminoacyl-tRNA synthetase and a tRNA.

ACKNOWLEDGMENT

We thank D. Horowitz, C. O'Day, M. Saks, and J. Sampson for advice and/or critical examination of the manuscript.

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