Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3463–3467, April 1992
Biochemistry

Switching $tRNA^{Gln}$ identity from glutamine to tryptophan

(tRNA specificity/aminoacyl-tRNA synthetase/nonsense suppression/evolution)

M. JOHN ROGERS*, TOSHIHARU ADACHI†, HACHIRO INOKUCHI†, AND DIETER SÖLL*

*Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511; and †Department of Biophysics, Kyoto University, Kyoto 606 Japan

Communicated by Sidney Altman, January 24, 1992 (received for review December 20, 1991)

ABSTRACT **The middle base (U35) of the anticodon of $tRNA^{Gln}$ is a major element ensuring the accuracy of aminoacylation by *Escherichia coli* glutaminyl-tRNA synthetase (GlnRS). An opal suppressor of $tRNA^{Gln}$ (su^+2UGA) containing C35 (anticodon UCA) was isolated by genetic selection and mutagenesis. Suppression of a UGA mutation in the *E. coli fol* gene followed by N-terminal sequence analysis of purified dihydrofolate reductase showed that this tRNA was an efficient suppressor that inserted predominantly tryptophan. Mutations of the 3–70 base pair (U70 and A3U70) were made. These mutants of su^+2UGA are less efficient suppressors and inserted predominantly tryptophan *in vivo*; alanine insertion was not observed. Mutations of the discriminator nucleotide (A73, U73, C73) result in very weak opal suppressors. Aminoacylation *in vitro* by *E. coli* TrpRS of $tRNA^{Gln}$ transcripts mutated in the anticodon demonstrate that TrpRS recognizes all three nucleotides of the anticodon. The results show the interchangeability of the glutamine and tryptophan identities by base substitutions in their respective tRNAs. The amber suppressor (anticodon CUA) $tRNA^{Trp}$ was known previously to insert predominantly glutamine. We show that the opal suppressor (anticodon UCA) $tRNA^{Gln}$ inserts mainly tryptophan. Discrimination by these synthetases for tRNA includes position 35, with recognition of C35 by TrpRS and U35 by GlnRS. As the use of the UGA codon as tryptophan in mycoplasma and in yeast mitochondria is conserved, recognition of the UCA anticodon by TrpRS may also be maintained in evolution.**

The anticodon of tRNA is critical for recognition by aminoacyl-tRNA synthetases (1–4). For *Escherichia coli* glutaminyl-tRNA synthetase (GlnRS) genetic, biochemical, and biophysical experiments have shown that all three anticodon bases are important. The significance of uridine as the middle base of the anticodon (U35) was first suggested when it was determined that the amber suppressor su^+7 (derived from $tRNA^{Trp}$ by a single mutation, C → U35) was mischarged with glutamine *in vitro* (5) and *in vivo* (6, 7). Chemical modification of the uridine derivative in position 34 of $tRNA_1^{Gln}$ (8) led to severe impairment of charging, while *in vitro* mutagenesis and subsequent aminoacylation of tRNA transcripts (9) showed the importance of all three nucleotides of the anticodon for recognition. The crystal structure of the GlnRS–$tRNA^{Gln}$ complex illustrates that each of the anticodon nucleotides is recognized by a binding pocket formed by the specific protein–tRNA interaction (10).

In light of these findings it was surprising that an opal suppressor of $tRNA^{Gln}$ (with C in position 35) was isolated by *in vivo* genetic selection (11). Thus, we wondered whether glutamine was still charged to this tRNA. This was even more in question because, in addition to the expected anticodon change, the tRNA had another mutation at position 70 of its acceptor stem. This C → U70 mutation creates a G3·U70 base pair, which is important for recognition by AlaRS (12, 13), and thus the mutated opal suppressor may not be aminoacylated with glutamine. However, suppression *in vivo* is a powerful way of determining the effect of competition in the aminoacylation of tRNA (14), which is important for the specificity of aminoacylation (14, 15). The loss of a major element for glutamine recognition (U → C35) can then be studied with opal suppressors *in vivo*, as U35 is a dominant feature for recognition of amber suppressors by GlnRS (16). Mutants of the *supE*/su^+2 amber suppressor $tRNA^{Gln}$ all retain aminoacylation by glutamine to >95% (J. Sherman and K. Rogers, personal communication). Competition *in vivo* may result in the opal suppressor $tRNA^{Gln}$ being mischarged, or being inactive if other synthetases do not share the recognition elements for the tRNA (17).

We have found that the active $tRNA^{Gln}$ opal suppressor SU^+2UGA‡ is mischarged with tryptophan at high efficiency *in vivo*. Aminoacylation by TrpRS *in vitro*, however, demonstrates the requirement for all three nucleotides (CCA) of the anticodon, as a transcript of $tRNA^{Gln}G1$ (CCA anticodon) is a much better substrate than this RNA with the opal (UCA) anticodon. These data confirm that the recognition of the anticodon is important for both TrpRS and GlnRS.

MATERIALS AND METHODS

Strains. *E. coli* LS653 (11) and XAC strains were described previously (16). Strain KL2576 was a gift of E. Murgola (University of Texas). Strains CSH39*recA* (18, 19) and CJ236 were used for *in vitro* mutagenesis, and DH5α for cloning.

***In Vivo* Isolation and DNA Sequencing of Temperature-Resistant su^+2UGA Mutants.** The hybrid λ/φ80 transducing phage (h^{80}cI857psu^+2UGA U70) was crossed with λ*imm*21 to replace the *c*I857 marker with the *imm*21 (cI^+) marker. The resultant phage (h^{80}*imm*21su^+2UGA U70) was mutagenized by preparing phage stocks with the soft agar containing 100 μl of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1 mg/ml) and the indicator bacteria CA274 (11). Mutagenized phage gave about 3–5% clear plaques.

More efficient UGA suppressors were isolated by plating the mutagenized h^{80}*imm*21su^+2UGA U70 phage on strain LS653 on M9 minimal lactose plates and incubating at 32°C for 3 days. Larger colonies were isolated and phage were prepared from these. A mutant from this phage was also selected that showed suppressor activity at 42°C. DNA prepared from the hybrid phages carrying the mutant UGA suppressors was digested with *Hin*cII and subjected to electrophoresis in low-melting agarose. The fragments of 150–300 base pairs were extracted from the gel slice and recloned in the *Hin*cII site of pUC18, selecting for ampicillin-resistant, Lac^+, Leu^+ transformants of LS653. DNA sequence was

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GlnRS, glutaminyl-tRNA synthetase; DHFR, dihydrofolate reductase.

‡su^+2UGA refers to the opal suppressor derived from $tRNA_2^{Gln}$, with mutations indicated by, for example, su^+2UGA C39 [change of pseudouridine (Ψ) to cytidine at position 39].

determined by dideoxy chain-termination, with double-stranded DNA as template.

***In Vitro* Mutagenesis of su$^+$2 and Mutants for *in Vitro* Transcription.** A synthetic gene for the amber suppressor derived from $tRNA_2^{Gln}$ (su$^+$2/*supE*), cloned on M13mp18 was used for *in vitro* mutagenesis (9, 19). An oligonucleotide complementary to the anticodon TCA was annealed to single-stranded DNA. The resulting opal suppressor mutants were recloned on plasmid pGFIB (20) for constitutive expression. For *in vitro* transcription, mutants of a synthetic $tRNA^{Gln}G1$ gene, with a G at position 1, were made by *in vitro* mutagenesis. A synthetic gene for *E. coli* $tRNA^{Trp}$ was constructed in pUC18, with deletion of the first nucleotide so that transcription begins with G2 ($tRNA^{Trp}\Delta A1$). Transcription of a *Bst*NI digest of plasmid DNA by T7 RNA polymerase was under conditions described previously (21). The RNA was purified by electrophoresis in denaturing polyacrylamide gels and, after elution from the gel, by DEAE-cellulose chromatography. After purification, transcripts of $tRNA^{Gln}G1$ still contained about 10–20% of a product one nucleotide shorter (shown by electrophoresis in denaturing polyacrylamide gels; data not shown). The $tRNA^{Trp}\Delta A1$ transcript appeared homogeneous after purification.

Suppression Assays. β-Galactosidase activity was assayed (18) with XAC strains and strain LS653 containing the opal suppressors either on a plasmid or as lysogens of mutants of $h^{80}imm21$su$^+$2UGA. Overnight cultures grown at the appropriate temperature (see Table 2) were inoculated into fresh M9 medium supplemented with 0.2% glycerol, 0.2% Casamino acids, thymine (30 μg/ml), tryptophan (30 μg/ml), and vitamin B1 (1.0 μg/ml). The cultures were shaken at the temperatures indicated until midlogarithmic phase, and then isopropyl β-D-thiogalactopyranoside was added (1 mM). After shaking for 1 hr, the cultures were chilled on ice and treated with toluene. The cultures were assayed for the rate of hydrolysis of *o*-nitrophenyl β-D-galactopyranoside, and units of β-galactosidase were calculated (18).

Purification of Dihydrofolate Reductase (DHFR). Strain YN2970 (22), mutated in release factor 2, was used for expression of DHFR suppressed by su$^+$2UGA suppressors (20, 23). DHFR was purified from 3 liters of culture from strains transformed with two compatible plasmids; containing the suppressor tRNA on a pGFIB derivative and a pACYC184 derivative, pD3op, containing the *E. coli fol* gene with a UGA codon at position 3 (17). For suppressors of >5% efficiency, the purified DHFR was sufficient for sequencing. Suppressors <5% efficient gave DHFR of low yield and below detectability for reliable sequencing. N-terminal amino acid sequence was determined by the Protein and Nucleic Acid Chemistry Facility, Yale University Medical School. The lower yield of phenylthiohydantoin-derivatized tryptophan (≈20%; K. Stone, personal communication) was taken into account in calculating the total; amino acids present at <5% were not considered significant. The strain YN2970/pD3op with no opal suppressor did not give sufficient DHFR for sequencing (data not shown); readthrough of UGA codons by $tRNA^{Trp}_{CCA}$ (24) is at a low level under these conditions and not adequate to give DHFR for sequencing.

Purification of *E. coli* TrpRS and Aminoacylation Assays. Strain DH5α was transformed with pCH6, a plasmid carrying the *E. coli trpS* gene (25), and used for purification of TrpRS as described (26). The *in vivo E. coli* $tRNA^{Trp}$ was purchased from Subriden RNA (Rolling Bay, WA). *In vitro* aminoacylation assays by TrpRS were carried out at pH 7.0 at 37°C (27), and the catalytic parameters of TrpRS for the $tRNA^{Gln}G1$ transcripts were determined under the conditions described for $tRNA^{Trp}$. However, parameters were determined at pH 7.0; although the optimum pH for TrpRS activity was determined at pH 8.8 (27), rather than "bias" aminoacylation toward tryptophan charging at this pH, conditions for glutamine charging (28) were chosen. The assay mixture (0.5 ml) contained 100 mM sodium cacodylate buffer (pH 7.0), 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM [^{14}C]tryptophan, and 0.01–1.0 μg of TrpRS, depending on the tRNA substrate. The transcripts were assayed over the range 0.1–10 μM. Aliquots taken at various time points were spotted on Whatman 3MM filter discs, presoaked in 10 mM tryptophan. The discs were washed and radioactivity was measured by scintillation counting (28).

RESULTS

Opal Suppressor $tRNA^{Gln}$ Mutants. The opal suppressor su$^+$2UGA derived by genetic selection contained an additional mutation (C → U70) in the acceptor stem (Fig. 1 *Left*) (11). To determine the properties of $tRNA^{Gln}$ opal suppressors without this additional U70 mutation or containing other mutations important for the identity of the tRNA, we generated these suppressors from characterized $tRNA^{Gln}$ amber suppressors (9). The opal suppressor $tRNA^{Gln}$ and the C → U70 and G → A3/C → U70 mutants were made by *in vitro* mutagenesis (Fig. 1 *Left*). Position 73 of tRNA is important for tRNA identity and has been termed the discriminator nucleotide (29). For this reason, G73 was mutated to all other nucleotides in the context of the glutamine opal suppressor.

The opal suppressor isolated *in vivo* was temperature-sensitive for suppression of the *lacZ659*(UGA) mutation in strain LS653 (Table 1). Therefore we wanted to select *in vivo* for opal suppressor $tRNA^{Gln}$ derivatives that were temperature-resistant for suppression. Temperature-resistant revertants of $h^{80}imm21$su$^+$2UGA U70 were isolated by suppression of *lacZ659*(UGA) in strain LS653 at various temperatures. DNA sequencing showed that the mutation responsible for the temperature-resistant phenotype in the tRNA was Ψ → C39 (data not shown).

Suppression of the *lacZ659*(UGA) Mutation. Suppression of *lacZ659*(UGA) was used to characterize mutants of the $tRNA^{Gln}$ opal suppressor. The temperature-resistant revertant su$^+$2UGA C39U70 suppresses at 30°C and 37°C (Table 1), whereas su$^+$2UGA C39 suppresses at 42°C as well. As discussed below, the role of this mutation is to increase suppressor efficiency, consistent with previous results for mutations outside the anticodon (30, 31). Mutations outside the anticodon of su$^+$2 amber suppressor have also been found that probably increase suppressor efficiency (32, 33) and are

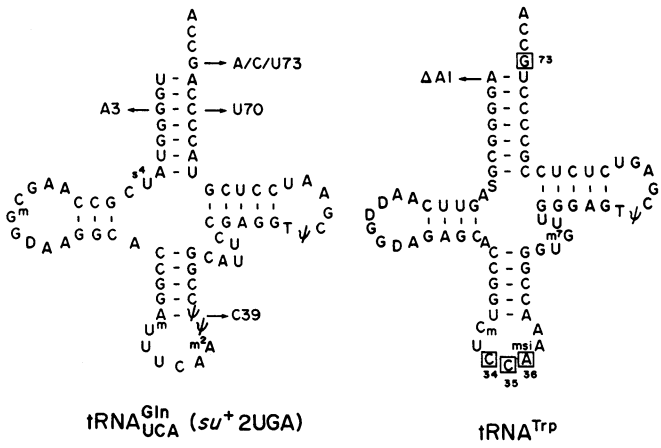

FIG. 1. (*Left*) Opal suppressor su$^+$2UGA derived from $tRNA_2^{Gln}$. Arrows indicate the positions of mutations made in the opal suppressor *in vitro* and *in vivo*, as discussed in the text. The modified nucleotides are inferred from the sequence of $tRNA_2^{Gln}$. (*Right*) *E. coli* $tRNA^{Trp}$, with boxed nucleotides showing the identity elements identified in this study. Arrow indicates the $tRNA^{Trp}\Delta A1$ transcript deleted so that transcription begins with G2.

Table 1. Suppression in strain LS653 by mutants of su$^+$2UGA

Suppressor	*lacZ659*(UGA) 30°C	37°C	42°C	β-Galactosidase activity, units (32°C)
h^{80}*imm*21su$^+$2UGA U70	+	−	−	9.3
h^{80}*imm*21su$^+$2UGA C39U70	+	+	−	96.0
h^{80}*imm*21su$^+$2UGA C39	+	+	+	220.0
psu$^+$2UGA	+	+	+	160.0
psu$^+$2UGA U70	+	±	±	31.0
psu$^+$2UGA A3U70	+	+	±	50.0
psu$^+$2UGA A73	+	±	−	*
psu$^+$2UGA C73	+	±	−	*
psu$^+$2UGA U73	+	±	−	*

Suppression was determined by growth (up to 3 days) on selective plates at the temperatures indicated. To test complementation by phage, a loop of phage (≈5 × 10^9 phage per ml) was cross-streaked against LS653 [*lacZ659*(UGA)]. +, Growth; −, no growth; ±, weak growth. β-Galactosidase activity was assayed (*Materials and Methods*) and is expressed in Miller units. h^{80}*imm*21su$^+$2UGA is the opal suppressor on phage; psu$^+$2UGA indicates the opal suppressor cloned on plasmid pGFIB. *, Values were not above that for the strain without opal suppressor (4.4 units).

not mischarged by the DHFR assay (34). It is not clear why an opal suppressor with changes only in the anticodon was not isolated by *in vivo* selection. As shown below, apart from suppressor efficiency, su$^+$2UGA and su$^+$2UGA C39 are indistinguishable.

The opal suppressor su$^+$2UGA derived from tRNA^{Gln} by *in vitro* mutagenesis suppressed *lacZ659*(UGA) similarly to the su$^+$2UGA C39 suppressor selected *in vivo* (Table 1). In addition, the su$^+$2UGA U70 and su$^+$2UGA A3U70 mutants suppressed *lacZ659*(UGA) in a somewhat temperature-sensitive manner (Table 1). Mutants of the discriminator nucleotide (su$^+$2UGA A/C/U73) of su$^+$2UGA were extremely weak suppressors that were also temperature-sensitive (Table 1).

The assay of β-galactosidase activity from the suppression of *lacZ659*(UGA) correlated with the suppression seen at the temperatures tested (Table 1). The su$^+$2UGA U70 suppressor gave the lowest β-galactosidase activity, whereas the C39 mutation increased the activity in the strain suppressed by both su$^+$2UGA C39 and su$^+$2UGA C39U70. For the opal suppressor mutants derived by *in vitro* mutagenesis, the su$^+$UGA suppressor showed the highest level of suppression, whereas both su$^+$UGA U70 and su$^+$UGA A3U70 were weaker suppressors. The difference between psu$^+$2UGA U70 (on plasmid pGFIB) and su$^+$2UGA U70 (on phage) may be due to higher plasmid copy number leading to elevated levels of expression. Mutants of the discriminator nucleotide (su$^+$2UGA A/C/U73) of su$^+$2UGA gave low levels of β-galactosidase that were not above the level of LS653 with no opal suppressor (su$^-$).

Suppressor Efficiency. The *lacI–Z* fusion system has been used to determine suppressor efficiency by measuring the percentage of readthrough at a termination codon in the *lacI* gene fused to *lacZ* (31). β-Galactosidase activity is then proportional to suppression of *lacI*, with generally little effect of the amino acid inserted by the suppressor. A number of UGA codons in *lacI* were used, as the surrounding sequence (or context) has an effect on the efficiency of suppression of the termination codon, particularly with the su$^+$9 tRNA^{Trp} opal suppressor (35). Also, there is background suppression of opal mutations in su$^-$ strains, presumably caused by readthrough by tRNA^{Trp} (24).

The strains XAC U4, U6, and U7 are isogenic with termination codons at different places in *lacI*; suppression of the U4 codon gives the highest values for suppressor efficiency, whereas U7 is poorly suppressed in most cases and U6 gives intermediate values for suppression. The values, as determined by β-galactosidase assays (18), are converted to a percentage with 100% defined as the β-galactosidase activity from strain XACΔ14 with a *lacI–Z* fusion but no termination codon in *lacI* (31).

The results (Table 2) show that su$^+$2UGA is 12% efficient in strain U4 but only 0.6% efficient in strain U7 (this is below the value for the su$^-$ strain, for reasons that are not clear) and thus has a Lac$^-$ phenotype in strain U7. The su$^+$2UGA C39 suppressor is of comparable efficiency in strain U4 but is more efficient than su$^+$2UGA in suppression of U6 and much more efficient in U7. Therefore, in codon contexts that are difficult to suppress, the su$^+$2UGA C39 and su$^+$2UGA C39U70 mutants are more efficient suppressors. The su$^+$2UGA U70 suppressor is less efficient (3.6–3.8%) in U4, with the difference between phage- and plasmid-encoded tRNA genes probably due to differences in levels of expression and/or modification. su$^+$2UGA A3U70 is also a weaker suppressor than su$^+$2UGA in all the contexts tested. Likewise, all the position 73 mutants of su$^+$2UGA showed weak, temperature-sensitive suppression of U4, although the su$^+$2UGA U73 was marginally the most active, and showed very weak growth on lactose minimal medium at 30°C (data not shown). The su$^+$2UGA U73 suppressor (GLNU73) had been described as being inactive (23). Because the su$^+$2UGA N73 mutant suppressors had a marginal Lac$^+$ phenotype, they were not studied in detail.

Amino Acid(s) Inserted by the Opal Suppressors. The spectrum of suppression by a number of amino acid substitutions in the α subunit of tryptophan synthetase (*trpA* gene product) has been studied extensively (36). At position 15, the wild-type amino acid is lysine, but substitution by glutamine (and a number of other amino acids; ref. 36) leads to a Trp$^+$ phenotype. Thus, an opal suppressor inserting glutamine at *trpA*(UGA15) gives Trp$^+$. However, an opal suppressor inserting tryptophan, or other amino acids at a reduced efficiency, will give a Trp$^-$ phenotype. The suppression of *trpA*(UGA15) then provides an assay for insertion of glutamine by the opal suppressors derived from su$^+$2 (Table 1). While su$^+$2UGA and su$^+$2UGA C39 suppress *trpA*(UGA15), all the other mutants of su$^+$2UGA do not suppress this opal mutation, either because they insert tryptophan (or some other amino acid not compatible with tryptophan synthetase activity), or because they have reduced suppressor efficiency, or both.

The amino acid specificity of a suppressor tRNA can be determined by purification and N-terminal sequencing of *E. coli* DHFR expressed from an inducible promoter, with an opal termination codon at codon 3 and an additional change at codon 4 (Met → Leu) to distinguish mutated from wild-type DHFR (23). The DHFR is expressed from a plasmid (pD3op)

Table 2. Efficiency of suppression by mutants of su$^+$2UGA

Suppressor	% suppression U4	U6	U7
su$^-$	0.9	3.8	1.3
h^{80}*imm*21su$^+$2UGA U70	3.8	6.0	3.3
h^{80}*imm*21su$^+$2UGA C39U70	6.4	11.6	4.0
h^{80}*imm*21su$^+$2UGA C39	4.4	10.8	6.0
psu$^+$2UGA	12.0	8.8	0.6
psu$^+$2UGA U70	3.6	3.6	0.4
psu$^+$2UGA A3U70	5.2	4.4	0.4
psu$^+$2UGA A73	3.5	*	*
psu$^+$2UGA C73	3.3	*	*
psu$^+$2UGA U73	4.5	*	*

Strains U4, U6, and U7 were grown at 32°C and β-galactosidase was assayed. Suppression is normalized to 100% (≈260 units) from strain XACΔ14. *, not determined; the values were not significantly above background (su$^-$).

compatible with the plasmid carrying the suppressor tRNA gene. To increase the suppressor efficiency and thus the yield of suppressed protein, purification was carried out in a strain mutated in release factor 2 (22). DHFR from su$^+$2UGA and su$^+$2UGA A3U70 has tryptophan at position 3 (88% and 90%, respectively; Table 3). The remaining 12% and 9%, respectively, is glutamine (including glutamate, which results from spontaneous deamidation). In the case of DHFR purified from the weaker su$^+$2UGA U70 suppressor, >90% of the signal was tryptophan (Table 3), with other amino acids present at <5%, including the wild-type amino acid at this position (serine). The opal suppressors derived from tRNA^{Gln} are then efficient, tryptophan-inserting tRNAs, presumably now being mischarged by *E. coli* TrpRS.

***In Vitro* Aminoacylation of tRNA Species.** To test the contribution of individual nucleotides of the anticodon to TrpRS recognition, anticodon mutants of tRNA^{Gln}G1 (the transcript initiates with the G at position 1 instead of U) were made for *in vitro* transcription by T7 RNA polymerase. The anticodon of tRNA$_2^{Gln}$ is CUG; the C35 mutant results in the anticodon CCG, the U34C35A36 mutant gives the opal anticodon UCA, whereas the C35A36 mutant has the anticodon CCA corresponding to tRNA^{Trp}. As a control, a gene for *E. coli* tRNA^{Trp} was constructed for *in vitro* transcription with T7 RNA polymerase (Fig. 1 *Right*); A1 was deleted so that transcription initiates with G2 (tRNA^{Trp}ΔA1). Aminoacylation of the *in vitro* transcripts was compared with that of *in vivo* purified tRNA^{Trp} (Table 4). The *in vivo* purified tRNA^{Trp} was aminoacylated with kinetic parameters similar to those described previously (27). The tRNA^{Trp}ΔA1 transcript was aminoacylated by TrpRS with a relative V/K_m ratio 2.5 times lower than *in vivo* tRNA^{Trp}; as has been found for other systems, modified nucleotides in tRNA help maintain the fidelity of aminoacylation by ensuring a tighter conformation of the tRNA (21, 37, 38). The first nucleotide of tRNA^{Trp} does not play a major role in recognition by TrpRS, as this nucleotide is deleted in the transcript. This is also the case for GlnRS recognition of tRNA^{Gln} (9). The tRNA^{Gln}G1 transcript that is the best substrate for TrpRS is one with the CCA anticodon (tRNA^{Gln}G1C35A36; Table 4) which has a V/K_m almost 10 times lower than tRNA^{Trp}ΔA1. However, since both of the kinetic parameters for the reaction are affected, other nucleotides in the tRNA aside from the major identity elements in the anticodon contribute to tryptophan identity. Surprisingly, the tRNA^{Gln}G1C35 (with the anticodon CCG) is the next-best substrate for TrpRS, followed by tRNA^{Gln}G1UCA. Therefore, although the opal suppressor derived from tRNA^{Gln} is efficiently mischarged by tryptophan *in vivo* (Table 3), *in vitro* tRNA^{Gln}G1UCA is not a good substrate. Although the first nucleotide (G1) may act as an antideterminant for TrpRS recognition, other reasons for this discrepancy are discussed below. The tRNA^{Gln}G1 (anticodon CUG) was, as expected, a very poor substrate for TrpRS, and kinetic parameters could not be accurately determined. The C35 and A36 single mutants of tRNA^{Gln}G1 are very poor substrates for GlnRS *in vitro* (9); kinetic parameters for aminoacylation by GlnRS of multiple anticodon mutants could not be obtained accurately (data not shown).

Table 3. *In vivo* incorporation of amino acids into DHFR by psu$^+$2UGA and mutants

Opal suppressor	Amino acids (%)
psu$^+$2UGA	Trp (88), Gln/Glu (12)
psu$^+$2UGA A3U70	Trp (90), Gln/Glu (9)
psu$^+$2UGA U70	Trp (90)*

*Estimated from small yield of amino acid at the site of the UGA codon; other amino acids were not present at ≥5%.

Table 4. Aminoacylation with TrpRS

tRNA	K_m, μM	V, μmol·min^{-1}·mg^{-1}	V/K_m (relative)
tRNA^{Trp} (*in vivo*)	0.29	4.5	2.5
tRNA^{Trp}ΔA1	0.37	2.3	1.0
tRNA^{Gln}G1*	94.0	0.0015	$<1.0 \times 10^{-5}$
tRNA^{Gln}G1C35	1.93	0.028	2.3×10^{-3}
tRNA^{Gln}G1U34C35A36	5.52	0.0043	1.3×10^{-4}
tRNA^{Gln}G1C35A36	1.66	1.2	0.12

Conditions for aminoacylation and determination of the kinetic constants were as described in *Materials and Methods*. The tRNA^{Trp} (*in vivo*) was isolated from cells; the other tRNAs were unmodified transcripts.

*Catalytic parameters could not be accurately determined under the conditions described.

DISCUSSION

tRNA Identity and the Trp/Gln System. The nucleotides that determine the specific recognition of tRNA by its cognate synthetase are termed the identity elements (16). This recognition includes productive interactions with the cognate synthetase and negative interactions to block the recognition of non-cognate synthetases. The mischarging by tryptophan of opal suppressors derived from tRNA^{Gln} suggests that the identity of tRNA^{Trp} involves anticodon recognition by TrpRS (Fig. 1 *Right*). This is confirmed by *in vitro* aminoacylation by TrpRS of tRNA^{Gln} transcripts (Table 4); tRNA^{Gln}G1 with the anticodon CCA is a good substrate for aminoacylation by TrpRS but is very poorly aminoacylated by GlnRS. Aminoacylation of tRNA^{Trp} transcripts also indicates anticodon recognition by TrpRS (51). It is still a formal possibility that the anticodon changes in tRNA^{Gln} block the recognition by GlnRS (and by "default" the tRNA is charged by TrpRS *in vivo*) rather than enhance productive interaction with TrpRS. If this is the case, the UCA anticodon is a negative determinant for GlnRS recognition rather than a positive element for TrpRS recognition. However, the efficiency of suppression argues against this, as efficient suppression implies efficient aminoacylation of the mutated tRNA (the level of incorporation of tryptophan *in vivo* is 88% with 0.6–12% efficiency of readthrough). There may also be discrimination by elongation factor Tu against tryptophan-charged tRNA (39) to favor incorporation of glutamine-charged tRNA by the translational apparatus, as this may account for the discrepancy for the mischarging of the tRNA^{Trp} amber suppressor (su$^+$7) *in vivo* and *in vitro* (39). Therefore, the level of mischarging detected by protein sequencing (Table 3) may represent a lower value than the actual level of misaminoacylation by TrpRS of su$^+$2UGA observed *in vivo*. The *in vitro* aminoacylation by TrpRS shows a requirement for recognition of all three anticodon nucleotides, as the tRNA^{Gln}G1 transcript with the anticodon (UCA) corresponding to an opal suppressor is a poor substrate for TrpRS, even though *in vivo* the opal suppressor is an efficient suppressor. This is analogous to the amber suppressor derived from tRNA^{Gln}; the mutation G → A36 has a large effect on charging *in vitro* (9) but the mutant is an efficient glutamine-inserting tRNA *in vivo* (su$^+$2/*supE*). This is probably a reflection of the sensitivity of the *in vivo* suppression compared with aminoacylation *in vitro*.

The discrimination between TrpRS and GlnRS includes recognition of the middle position of the anticodon, and TrpRS may recognize this position in a similar manner as GlnRS (10), as they are both class I synthetases (40). However, TrpRS is a 74-kDa α_2 dimer, while GlnRS is a 63-kDa monomer; the apparent similarity in recognition of both the anticodon and acceptor stem by the two enzymes suggests that TrpRS may bind tRNA across the surface of the dimer. This question awaits the structural analysis of TrpRS (41).

Is the Acceptor Stem of tRNA the Primary Site of Interaction by Synthetases? It is surprising that the opal suppressor derived from $tRNA^{Gln}$ retains glutamine acceptance *in vivo*, as all three anticodon nucleotides are specifically recognized by GlnRS (10). This implies that identity elements in the acceptor end of $tRNA^{Gln}$ are a major site of interaction with the synthetase, with anticodon recognition serving to modulate the specificity. This is supported by results from the 3–70 mutants and 73 mutants of su^+2UGA. The recognition of the 3–70 base pair may not be critical for TrpRS, and competition with GlnRS, which specifically recognizes the G3·C70 base pair (19), results in charging with GlnRS, and thus reduced suppressor efficiency of su^+2UGA A3U70 and su^+2UGA U70. In addition, G73 forms a specific intramolecular interaction in $tRNA^{Gln}$ that contributes to glutamine identity (42); this may be the case for $tRNA^{Trp}$ identity (Fig. 1 *Right*) as the position 73 mutants of su^+2UGA are extremely weak suppressors. It is possible to divide tRNAs as opal suppressors into classes based on the discriminator nucleotide; with G73 the opal suppressor is primarily recognized by TrpRS, while with A73 or G73 (and A20) (17) recognition is by ArgRS, and with U73, by GlyRS (23, 36).

That the acceptor stem plays a major role in tRNA recognition is supported by specific aminoacylation of minihelices derived from the acceptor stem of $tRNA^{Ala}$ and $tRNA^{His}$ (e.g., ref. 43). Moreover, mutants of GlnRS with altered acceptor-stem recognition show relaxed tRNA specificity (44). Transplanting the proposed tryptophan identity elements into, for example $tRNA^{fMet}$ (45), may reveal the contribution of the acceptor stem to $tRNA^{Trp}$ *in vivo*.

Evolution of TrpRS Identity. The UGA codon (usually a termination codon) has been found in a number of cases to direct insertion of tryptophan. In *E. coli*, two opal suppressors derived from $tRNA^{Trp}$ have been isolated. One suppressor (su^+7UGA) has the expected mutation in the anticodon (46), whereas the other suppressor (su^+9) has a G → A24 mutation in the D stem (47). The insertion of tryptophan at UGA codons may be conserved, as mycoplasma, which are related to Gram-positive eubacteria (48), have two $tRNA^{Trp}$ genes (49); the minor (≈15%) $tRNA^{Trp}$ has the usual CCA anticodon, whereas the major $tRNA^{Trp}$ possesses a UCA anticodon. The UGA codon may also be read as tryptophan in eukaryotic mitochondria, as the gene for $tRNA^{Trp}_{UCA}$ is found, for example, in yeast mitochondria (50). Assuming that TrpRS recognition is conserved in these cases, the identity of $tRNA^{Trp}$ may also be conserved in evolution.

We are grateful to C. Carter, Jr., W. McClain, J. Miller, E. Murgola, Y. Nakamura, and W. Studier for the generous gifts of strains and plasmids. We thank J. Sherman for critical reading of the manuscript and C. Boyd for assistance with some of the experiments. This work was supported by a grant from the National Institutes of Health and by a grant-in-aid on "Diversity of the genetic code" from the Ministry of Education, Science, and Culture of Japan.

1. Zachau, H. G. (1964) *Hoppe-Seyler's Z. Physiol. Chem.* **336**, 176–188.
2. Deutscher, M. (1965) *Biochem. Biophys. Res. Commun.* **19**, 283–288.
3. Hayashi, H. & Miura, K. I. (1966) *Nature (London)* **209**, 376–378.
4. Deutscher, M. P. (1967) *J. Biol. Chem.* **242**, 3601–3607.
5. Yarus, M., Knowlton, R. & Soll, L. (1977) in *Nucleic Acid-Protein Recognition*, ed. Vogel, H. J. (Academic, New York), pp. 391–408.
6. Yaniv, M., Folk, W. R., Berg, P. & Soll, L. (1974) *J. Mol. Biol.* **86**, 245–260.
7. Celis, J. E., Coulondre, C. & Miller, J. H. (1976) *J. Mol. Biol.* **104**, 729–734.
8. Seno, T., Agris, P. F. & Söll, D. (1974) *Biochim. Biophys. Acta* **349**, 328–338.
9. Jahn, M., Rogers, M. J. & Söll, D. (1991) *Nature (London)* **352**, 258–260.
10. Rould, M. A., Perona, J. J. & Steitz, T. A. (1991) *Nature (London)* **352**, 213–218.
11. Inokuchi, H., Kondo, K., Yoshimura, M. & Ozeki, H. (1990) *Mol. Gen. Genet.* **223**, 433–437.
12. Hou, Y.-M. & Schimmel, P. (1988) *Nature (London)* **333**, 140–145.
13. McClain, W. H. & Foss, K. (1988) *Science* **240**, 793–796.
14. Swanson, R., Hoben, P., Sumner-Smith, M., Uemura, H., Watson, L. & Söll, D. (1988) *Science* **242**, 1548–1551.
15. Sherman, J. M., Rogers, M. J. & Söll, D. (1992) *Nucleic Acids Res.*, in press.
16. Normanly, J. & Abelson, J. (1989) *Annu. Rev. Biochem.* **58**, 1029–1049.
17. McClain, W., Foss, K., Jenkins, R. A. & Schneider, J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 9260–9264.
18. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
19. Rogers, M. J. & Söll, D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6627–6631.
20. Normanly, J., Ogden, R. C., Horvath, S. J. & Abelson, J. (1986) *Nature (London)* **321**, 213–219.
21. Sampson, J. R. & Uhlenbeck, O. C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1033–1037.
22. Kawakami, K., Inada, T. & Nakamura, Y. (1988) *J. Bacteriol.* **170**, 5378–5381.
23. McClain, W. H., Foss, K., Jenkins, R. H. & Schneider, J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6147–6151.
24. Hirsh, D. & Gold, L. (1971) *J. Mol. Biol.* **58**, 459–468.
25. Hall, C. V., van Cleemput, M., Muench, K. H. & Yanofsky, C. (1982) *J. Biol. Chem.* **257**, 6132–6136.
26. Beresten, S. F., Zargarova, T. A., Favorova, O. O., Rubikaite, B. I., Ryazanov, A. G. & Kisselev, L. L. (1989) *Eur. J. Biochem.* **184**, 575–581.
27. Joseph, D. R. & Muench, K. H. (1971) *J. Biol. Chem.* **246**, 7602–7609.
28. Hoben, P. & Söll, D. (1985) *Methods Enzymol.* **113**, 55–59.
29. Crothers, D. M., Seno, T. & Söll, D. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3063–3067.
30. Yarus, M., Cline, S., Raftery, L., Wier, P. & Bradley, D. (1986) *J. Biol. Chem.* **261**, 10496–10505.
31. Kleina, L. G., Masson, J.-M., Normanly, J., Abelson, J. & Miller, J. H. (1990) *J. Mol. Biol.* **213**, 705–717.
32. Bradley, D., Park, J. V. & Soll, L. (1981) *J. Bacteriol.* **145**, 704–712.
33. Yamao, F., Inokuchi, H. & Ozeki, H. (1988) *Jpn. J. Genet.* **63**, 237–249.
34. Yamao, F., Inokuchi, H., Normanly, J., Abelson, J. & Ozeki, H. (1988) *Jpn. J. Genet.* **63**, 251–258.
35. Miller, J. H. & Albertini, A. M. (1983) *J. Mol. Biol.* **164**, 59–71.
36. Murgola, E. J. (1985) *Annu. Rev. Genet.* **19**, 57–80.
37. Schulman, L. H. & Pelka, H. (1988) *Science* **242**, 765–768.
38. Giege, R., Florentz, C., Grosjean, H., Perret, V., Puglisi, J., Theobald-Dietrich, A. & Ebel, J.-P. (1990) *Biochimie* **72**, 453–461.
39. Knowlton, R. G., Soll, L. & Yarus, M. (1980) *J. Mol. Biol.* **139**, 705–720.
40. Eriani, G., Delarue, M., Poch, O., Gangloff, J. & Moras, D. (1990) *Nature (London)* **247**, 203–206.
41. Carter, C. W., Jr., & Coleman, D. E. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 2981–2983.
42. Rould, M. A., Perona, J., Söll, D. & Steitz, T. (1989) *Science* **246**, 1135–1142.
43. Francklyn, C. & Schimmel, P. (1989) *Nature (London)* **337**, 478–481.
44. Perona, J. J., Swanson, R. N., Rould, M. A., Steitz, T. A. & Söll, D. (1989) *Science* **246**, 1152–1154.
45. Pallanck, L. & Schulman, L. H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3872–3876.
46. Eggertsson, G. & Söll, D. (1988) *Microbiol. Rev.* **52**, 354–374.
47. Hirsh, D. (1971) *J. Mol. Biol.* **58**, 439–458.
48. Rogers, M. J., Simmons, J., Walker, R. T., Weisburg, W. G., Woese, C. R., Tanner, R. S., Robinson, I. M., Stahl, D. A., Olsen, G., Leach, R. H. & Maniloff, J. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1160–1164.
49. Yamao, F., Iwagami, S., Azumi, Y., Muto, A., Osawa, S., Fujita, N. & Ishihama, A. (1988) *Mol. Gen. Genet.* **212**, 364–369.
50. Martin, N. C., Pham, H. D., Underbrink-Lyon, K., Miller, D. L. & Donelson, J. E. (1980) *Nature (London)* **285**, 579–581.
51. Himeno, H., Hasegawa, T., Asahara, H., Tamura, K. & Shimizu, M. (1991) *Nucleic Acids Res.* **23**, 6379–6382.