Biochemistry

 Volume 31, Number 13 April 7, 1992

Accelerated Publications

Conversion of a Methionine Initiator tRNA into a Tryptophan-Inserting Elongator tRNA in Vivo†

Marie Pak, Leo Pallanck, and LaDonne H. Schulman*
Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, New York 10461
Received December 13, 1991; Revised Manuscript Received February 5, 1992

ABSTRACT: The role of the anticodon and discriminator base in aminoacylation of tRNAs with tryptophan has been explored using a recently developed in vivo assay based on initiation of protein synthesis by mischarged mutants of the *Escherichia coli* initiator tRNA. Substitution of the methionine anticodon CAU with the tryptophan anticodon CCA caused $tRNA^{fMet}$ to be aminoacylated with both methionine and tryptophan in vivo, as determined by analysis of the amino acids inserted by the mutant tRNA at the translational start site of a reporter protein containing a tryptophan initiation codon. Conversion of the discriminator base of $tRNA^{fMet}_{CCA}$ from A73 to G73, the base present in $tRNA^{Trp}$, eliminated the in vivo methionine acceptor activity of the tRNA and resulted in complete charging with tryptophan. Single base changes in the anticodon of $tRNA^{fMet}_{CCA}$ containing G73 from CCA to UCA, GCA, CAA, and CCG (changes underlined) essentially abolished tryptophan insertion, showing that all three anticodon bases specify the tryptophan identity of the tRNA. The important role of G73 in tryptophan identity was confirmed using mutants of an opal suppressor derivative of $tRNA^{Trp}$. Substitution of G73 with A73, C73, or U73 resulted in a large loss of the ability of the tRNA to suppress an opal stop codon in a reporter protein. Base pair substitutions at the first three positions of the acceptor stem of the suppressor tRNA caused 2–12-fold reductions in the efficiency of suppression without loss of specificity for aminoacylation of the tRNA with tryptophan. Base pair changes in $tRNA^{fMet}_{CCA}$/G73 at positions 1·72 and 3·70 to match sequences present in $tRNA^{Trp}$ allowed the mutant initiator tRNA to function as an efficient tryptophan-inserting elongator tRNA in vivo, as judged by the ability of the tRNA to complement a strain of *E. coli* carrying a temperature-sensitive $tRNA^{Trp}$ at the nonpermissive temperature.

Attachment of the appropriate amino acid to each tRNA molecule is a crucial step in the translation of genetic information. The highly accurate aminoacylation of tRNAs is catalyzed by aminoacyl-tRNA synthetases specific for each of the 20 amino acids. The amino acid acceptor specificity, or identity, of each tRNA is determined by a particular set of structural features that allows recognition by one synthetase and excludes recognition by all others. In the past few years, a variety of new approaches have been used to determine the location of these positive and negative identity elements in tRNAs as a step toward elucidating the mechanism of this fundamental process (Normanly & Abelson, 1989; Schimmel, 1989; Schulman, 1991).

The results of both in vivo and in vitro studies have indicated an important role for the anticodon in aminoacylation of many tRNAs [reviewed in Schulman (1991)]. The earliest in vivo evidence that anticodon bases play a role in the fidelity of aminoacylation of a tRNA was reported by Berg and coworkers. An amber suppressor derivative of *Escherichia coli* $tRNA^{Trp}$ containing an anticodon change from CCA to CUA was obtained by genetic selection and shown to insert both tryptophan and glutamine at the site of amber stop codons in vivo (Soll & Berg, 1969; Yaniv et al., 1974; Celis et al., 1976). Yarus et al. (1977) subsequently isolated the mutant tRNA and shows that the single anticodon base change simultaneously decreased its tryptophan and increased its glutamine acceptor activity. These in vitro results nicely explained the ability of the glutamine and tryptophan synthetases to suc-

†Supported by American Cancer Society Grant NP19T. M.P. was supported by NIH Training Grant 2T32GM07128, and L.P. was supported by NIH Training Grant T32GM07491. Partial salary support for L.H.S. was provided by NCI Grant P30CA13330.
* To whom correspondence should be addressed.

0006-2960/92/0431-3303$03.00/0

cessfully compete with each other for the mutant tRNA in vivo (Knowlton et al., 1980) and showed the importance of the middle anticodon base, C35, for recognition of $tRNA^{Trp}$ by its cognate synthetase and of U35 for tRNA substrate selection by the glutamine enzyme.

No information is presently available on the role of the other two anticodon bases in recognition of tRNAs by TrpRS[1] or on the location of important identity elements outside of the anticodon of $tRNA^{Trp}$. We have recently developed an in vivo assay to allow examination of the effects of anticodon base changes on tRNA identity, as well as the effects of mutations in other structural domains in the context of a tRNA containing a naturally occurring anticodon sequence (Chattapadhyay et al., 1990; Pallanck & Schulman, 1991). This assay utilizes derivatives of the *E. coli* initiator tRNA containing non-methionine anticodons to test for the effects of structural changes on in vivo aminoacylation. The identity of the $tRNA^{fMet}$ mutants is determined by analysis of the amino acid(s) inserted by each tRNA at a complementary initiation codon in the gene for dihydrofolate reductase. To date, this assay has been used to show that all three bases of the isoleucine anticodon GAU and the phenylalanine anticodon GAA are required for specific aminoacylation of $tRNA^{fMet}$ with isoleucine and phenylalanine, respectively, and that the two conserved bases of the valine anticodons UAC and GAC are required for in vivo aminoacylation of the tRNA with valine (Pallanck & Schulman, 1991). In addition, Varshney and RajBhandary (1990) have demonstrated that $tRNA^{fMet}$ containing the amber anticodon CUA inserts glutamine in vivo. In the present work, we have used the in vivo initiation assay to examine the structural features required for specific aminoacylation of $tRNA^{fMet}$ with tryptophan.

Materials and Methods

Expression Vectors. The $tRNA^{fMet}$ expression vector pHP12 and the method of construction of various $tRNA^{fMet}$ mutants have been described (Chattapadhyay et al., 1990). The vector used for expression of $tRNA^{Trp}$ derivatives (p184) was constructed using the *Pvu*II/*Bcl*I fragment of plasmid pACYC184 (Chang & Cohen, 1978). Transcription of tRNA genes was initiated from a synthetic *tac* promoter, inserted at the *Pvu*II site, upon induction with IPTG. The plasmid retained the tetracycline-resistance gene and the p15A origin of replication of the original vector. Construction of plasmid pLP5 containing various initiation codons at the translational start site of an N-terminally extended DHFR gene has been described elsewhere (Pallanck & Schulman, 1991). A plasmid (pMP5) containing a UGA termination signal at codon 3 of the DHFR gene was obtained by replacement of the *Xho*I/*Hin*dIII fragment of pLP5 with the appropriate annealed synthetic deoxyoligonucleotides.

DHFR Assay, Purification, and Sequencing. DHFR synthesized by initiation with $tRNA^{fMet}$ mutants was assayed in a DHFR-deficient *E. coli* strain as described before (Pallanck & Schulman, 1991). Opal suppression assays were similarly carried out in strain MP1, derived from strain PA414 (Ahrweiler & Frieden, 1988), by transfer of the F′ (*lacI*^{Q}, *proAB*$^{+}$) from JM109 (Yanisch-Perron et al., 1985), except that tetracycline (5 μg/mL) was substituted for chloramphenicol in the growth medium. DHFR protein was purified by methotrexate affinity chromatography followed by reverse-phase HPLC chromatography using a gradient of acetonitrile in 0.1% trifluoroacetic acid (Pallanck & Schulman, 1991). The purified protein was subjected to five cycles of Edman degradation on a gas-phase protein sequencer.

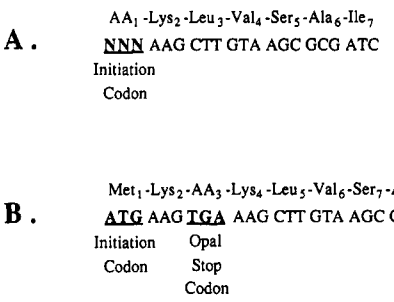

FIGURE 1: Sequence of the translational initiation site of the DHFR constructs used in these studies. (A) Construct used for measurement of the initiation activity of $tRNA^{fMet}$ mutants. (B) Construct used for measurement of opal suppressor activity. In both cases, the isoleucine corresponds to Ile2 of wild-type DHFR, and the sequence thereafter is identical in the wild-type and N-terminally extended proteins.

Northern Analyses. The intracellular levels of wild-type and mutant tRNAs were determined by Northern analysis as described by Smith and Yarus (1989). The levels of in vivo aminoacylation of specific tRNAs were determined by Northern analysis following isolation and gel electrophoresis of the tRNAs under acidic conditions, as described by Varshney et al. (1991a).

Complementation of an E. coli Strain Containing a Temperature-Sensitive Chromosomal $tRNA^{Trp}$. *E. coli* strain LS874 (W3110 *trpR trpA9605 his29 metE* $trpT_{ts}$; Yanofsky & Soll, 1977) was transformed with plasmids carrying various tRNA genes, plated on LB containing the appropriate antibiotic, and grown at 37 °C. Single colonies of fresh transformants were picked, grown overnight at 37 °C, diluted 1:100 with fresh medium, and grown to saturation at 37 or 42 °C. Diluted cultures grown at 37 °C were streaked on LB plates and incubated overnight at 37 or 42 °C.

Results

$tRNA^{fMet}$ Containing a Tryptophan Anticodon Inserts Tryptophan into DHFR. *E. coli* DHFR was used as a reporter protein to determine the amino acid(s) inserted by mutant derivatives of $tRNA^{fMet}$. The sequence of the wild-type protein was slightly modified by addition of five amino acids to the N-terminus (Pallanck & Schulman, 1991). The modified sequence contains a lysine residue at position 2 (Figure 1A), which blocks removal of the N-terminal amino acid in vivo (Ben-Bassat et al., 1987; Hirel et al., 1989).

The methionine ATG initiation codon of the plasmid-borne DHFR gene was changed to the tryptophan codon TGG. A derivative of $tRNA^{fMet}$ containing the complementary tryptophan anticodon CCA (Figure 2) was expressed from a compatible plasmid at a level comparable to that of endogenous tRNAs (Chattapadhyay et al., 1990). In the absence of the complementary initiator tRNA, little or no DHFR was produced (Table I). Expression of the mutant tRNA resulted in a 35-fold stimulation of DHFR synthesis. Isolation of the protein on a methotrexate affinity column (Normanly et al., 1986) followed by HPLC chromatography yielded two peaks of DHFR protein. Edman degradation of protein isolated from these peaks showed that the earlier eluting peak contains N-terminal methionine, while the other peak is initiated with tryptophan. No other N-terminal amino acid was detected.

[1] Abbreviations: $tRNA^{fMet}$, *Escherichia coli* initiator methionine tRNA; $tRNA^{fMet}_{CCA}$, $tRNA^{fMet}$ containing a CCA anticodon (similar notation is used for other tRNA anticodon derivatives); DHFR, dihydrofolate reductase; GlnRS, glutaminyl-tRNA synthetase; TrpRS, tryptophanyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; IPTG, isopropyl β-D-thiogalactopyranoside.

Table I: Synthesis of DHFR from Various Initiation Codons in the Presence and Absence of Complementary $tRNA^{fMet}$ Anticodon Mutants[a]

initiation codon 5′ → 3′	$tRNA^{fMet}$ anticodon 5′ → 3′	position 73	DHFR units +complementary $tRNA^{fMet}$	DHFR units −complementary $tRNA^{fMet}$	efficiency rel to AUG	N-terminal amino acids (%)
AUG	CAU	A	1085 ± 94	1109 ± 133	100	Met (100)
UGG	CCA	A	28 ± 4	0.8 ± 0.2	2.5	Met (60), Trp (40)
UGG	CCA	G	56 ● 7	0.8 ± 0.2	5.1	Trp only[b]
UGA	UCA	G	1.2 ± 0.2	0.5 ± 0.1	0.06	
UGC	GCA	G	1.1 ± 0.3	0.5 ± 0.1	0.06	
UUG	CAA	G	0.6 ± 0.2	0.6 ± 0.1	0	
CGG	CCG	G	25 ± 2	0.5 ● 0.1	2.2	mainly Gln[c]

[a] DHFR units are the average of at least three separate determinations. Efficiency relative to AUG is calculated after subtracting the DHFR units obtained in the absence of the complementary $tRNA^{fMet}$ for each codon. [b] See text. [c] The exact yield of Gln could not be determined due to extensive cyclization of N-terminal Gln to a derivative resistant to Edman degradation. The unblocked protein yielded 71% Gln, 18% Glu, and 11% Gly. The yield of Gly calculated on the basis of total protein is less than 3%. Glu is presumed to be derived by partial breakdown of Gln during the Edman analysis.

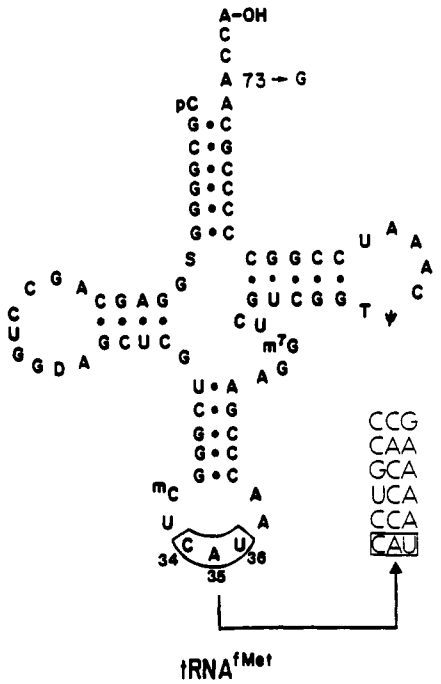

FIGURE 2: Structure of the *E. coli* initiator tRNA showing the anticodon mutants used in this work and the change in the discriminator base.

The Discriminator Base Plays a Major Role in Tryptophan Identity. As noted above, $tRNA^{fMet}_{CCA}$ is aminoacylated with both methionine and tryptophan in vivo, suggesting that the anticodon change has reduced the methionine and increased the tryptophan acceptor activity of the tRNA. In order to completely alter the identity of the tRNA, one additional base change was required at the "discriminator base" (Crothers et al., 1972) (position 73). The initiator tRNA contains A73 (Figure 2) while G73 is found in $tRNA^{Trp}$ (Figure 3). An A73 to G73 mutation was made in $tRNA^{fMet}$ containing the tryptophan anticodon to yield $tRNA^{fMet}_{CCA}$/G73. Expression of this tRNA in the presence of the reporter protein carrying a tryptophan initiation codon resulted in a 70-fold increase in DHFR synthesis over the level observed in the absence of the tRNA. The isolated protein eluted as a single peak on HPLC chromatography at the position corresponding to that of the tryptophan-initiated DHFR derivative (data not shown), and protein sequencing showed that tryptophan was the only detectable N-terminal amino acid (Table I). Examination of the in vivo level of aminoacylation of $tRNA^{fMet}_{CCA}$/G73 by Northern analysis of tRNAs on acid/urea polyacrylamide gels (Varshney et al., 1991a) showed that the tRNA was fully charged with tryptophan (data not shown).

The important role of the discriminator base in tryptophan identity was confirmed by examination of the effect of mutations at this site on the in vivo activity of an opal suppressor derivative of $tRNA^{Trp}$ (Figure 3A). A single base change in the anticodon of $tRNA^{Trp}$ from CCA to UCA allows insertion of Trp by the mutant tRNA at the site of UGA stop codons in vivo (Raftery et al., 1984). An opal codon was inserted at position 3 of the DHFR construct (Figure 1B), and the suppressor activity of various $tRNA^{Trp}_{UCA}$ derivatives was determined by measurement of the amount of DHFR produced in the presence of the plasmid-borne tRNAs. Mutations at position 73 were seen to virtually abolish the activity of the suppressor tRNA (Table II).

All Three Anticodon Bases Are Important for the Tryptophan Identity of Mutant Initiator tRNAs. In order to examine the role of individual anticodon bases in the in vivo aminoacylation of the initiator tRNA with tryptophan, single base changes were introduced into the anticodon of $tRNA^{fMet}_{CCA}$/G73. Substitution of the wobble base C34 with either G34 or U34 reduced the level of initiation of DHFR synthesis by the corresponding mutant tRNAs to near-background levels (Table I), indicating an important role for this site. Early in vivo and in vitro studies by others previously showed that conversion of the middle anticodon base (C35) of $tRNA^{Trp}$ to U35 produced a derivative containing dual tryptophan and glutamine identity (Celis et al., 1976; Yarus et al., 1977; Knowlton et al., 1980). We have confirmed the important role of this nucleotide by creating a different base substitution at this site in $tRNA^{fMet}_{CCA}$/G73. Conversion of C35 to A35 abolished the initiation activity of the tRNA (Table I). Substitution of the 3′-terminal anticodon nucleotide, A36, with G36 resulted in about a 2-fold decrease in the level of DHFR synthesis initiated by the mutant tRNA. Sequence analysis of the isolated protein showed that greater than 80% of the protein was refractory to Edman degradation. The unblocked protein yielded mainly glutamine, and no tryptophan, in cycle 1. N-Terminal glutamine residues are well-known to readily cyclize to pyrrolidone carboxyl derivatives that block the sequencing reaction (Smyth et al., 1963).

Base Pairs in the Acceptor Stem Contribute to the Efficiency but Not the Specificity of Tryptophan Insertion into DHFR by Tryptophan Opal Suppressor tRNA. The effect of changes in the first three base pairs of the acceptor stem on in vivo tryptophan acceptor activity was examined using the opal suppressor derivative of $tRNA^{Trp}$ (Figure 3A). Conversion of the wild-type A1·U72 sequence to either U1·A72 or G1·C72 produced about a 2-fold decrease in the level of suppression of an opal stop codon at position 3 of the DHFR reporter protein (Table II). Substitution of G2·C71 with C2·G71 produced about a 12-fold decrease in DHFR synthesis,

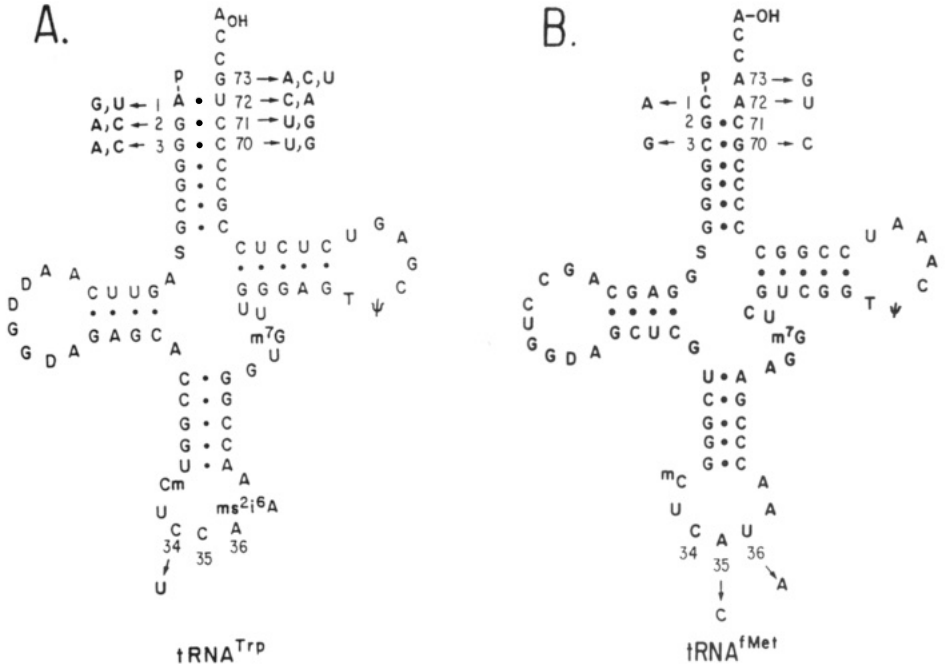

FIGURE 3: Structures of tryptophan-inserting elongator tRNAs. (A) Opal suppressor derivatives of $tRNA^{Trp}$ studied in this work. (B) Mutations in $tRNA^{fMet}$ used to convert the initiator tRNA into an efficient tryptophan-inserting elongator tRNA.

Table II: Effect of Mutations in the Acceptor Stem and Discriminator Base on the Efficiency of Suppression of an Opal Stop Codon in DHFR by $tRNA^{Trp}_{UCA}$ [a]

tRNA sequence		units of DHFR	amino acid inserted[c]	rel efficiency of Trp insertion
wild type	mutant			
G73		889 ± 81	Trp	100
	A73	16 ± 2	ND	0–2
	C73	1 ± 0.1[b]	ND	0–0.1
	U73	11 ± 1	ND	0–1
A1·U72	U1·A72	395 ± 74	Trp	44
	G1·C72	426 ± 44	Trp	48
G2·C71	C2·G71	67 ± 23	Trp	8
	A2·U71	174 ± 32	Trp	19
G3·C70	C3·G70	329 ± 52	Trp	37
	A3·U70	95 ± 31	Trp	11

[a] DHFR units are the average of six to nine separate determinations. The background level of DHFR synthesis in the absence of added tRNA (31 units) has been subtracted in each case. Suppression efficiency is relative to that of $tRNA^{Trp}_{UCA}$. In cases where protein has not been sequenced, the maximum efficiency given assumes 100% insertion of Trp and the minimum efficiency of 0 assumes no insertion. [b] Northern analysis indicated that the C73 mutant was present in the cell at a significantly lower level than the other discriminator base mutants tested. [c] In all of the sequenced proteins, no amino acid other than Trp was observed at a level ≥5% except for DHFR derived from the C2·G71 mutant. Protein from this mutant contained 10% of a second N-terminal sequence, Trp-Lys-Leu-Val-Ser, presumably derived by partial degradation of the initial product containing the sequence Met-Lys-Trp-Lys-Leu-Val-Ser. ND = not determined.

while a $tRNA^{Trp}_{UCA}$ mutant containing A2·U71 yielded 5-fold less protein than tRNA containing the wild-type acceptor stem sequence. Conversion of G3·C70 to C3·G70 or A3·U70 decreased the yields of DHFR 3-fold and 9-fold, respectively (Table II). N-Terminal sequence analysis of protein produced in the presence of each $tRNA^{Trp}_{UCA}$ derivative revealed that Trp was the only amino acid inserted by the mutant tRNAs.

Conversion of the Initiator $tRNA^{fMet}$ into an Efficient Tryptophan-Inserting Elongator tRNA. *E. coli* $tRNA^{fMet}$ is unable to participate in elongation of protein synthesis due to the presence of an unpaired C1·A72 sequence at the 5′ end of the acceptor stem (Seong & RajBhandary, 1987). In order to examine the ability of $tRNA^{fMet}_{CCA}$/G73 to function as a highly efficient tryptophan-inserting tRNA in vivo, we sought to determine whether a mutant derivative of the initiator tRNA containing a normal base pair at the 5′ end could substitute for the function of endogenous $tRNA^{Trp}$ in protein synthesis in rapidly growing cells. For this purpose, *E. coli* strain LS874 (Yanofsky & Soll, 1977) containing a mutation in the sole gene for $tRNA^{Trp}$ was utilized. This strain grows normally at 37 °C but is unable to grow at 42 °C due to production of a temperature-sensitive $tRNA^{Trp}$ that is degraded at the higher temperature (Eisenberg et al., 1979; Eisenberg & Yarus, 1980). A derivative of $tRNA^{fMet}$ was constructed that contained the tryptophan anticodon and discriminator base G73 and "Trp" A1·U72 and G3·C70 sequences in the acceptor stem (Figure 3B). The tryptophan and methionine tRNAs share the same sequence, G2·C71, at the second position of the acceptor stem.

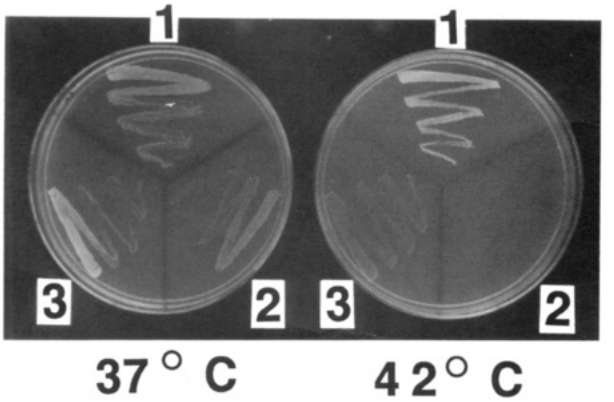

FIGURE 4: Complementation of an *E. coli* strain containing a temperature-sensitive endogenous $tRNA^{Trp}$ with plasmid-borne tRNAs. *E. coli* LS874 cells were grown at 37 °C (left) or 42 °C (right) as described under Materials and Methods. The sectored plate shows the growth of cells transformed with (1) $tRNA^{Trp}_{CCA}$, (2) $tRNA^{fMet}_{CAU}$, and (3) $tRNA^{fMet}_{CCA}$/G73/A1·U72/G3·C70.

Figure 4 shows that the plasmid-borne mutant $tRNA^{fMet}$ was able to complement the temperature-sensitive defect of strain LS874. A control experiment carried out with wild-type $tRNA^{fMet}$ failed to allow growth at 42 °C. Northern analysis

of tRNA isolated under acidic conditions indicated that the mutant tRNA was present at a level comparable to that of endogenous tRNAs and was fully aminoacylated in vivo (data not shown). Expression of a similar level of plasmid-borne $tRNA^{Trp}$ resulted in about 2-fold faster growth of the cells at 42 °C. The mutant initiator tRNA had no effect on the rate of growth of cells at 37 °C.

Discussion

Using an assay based on initiation of protein synthesis by mutants of $tRNA^{fMet}$, we have examined the role of anticodon bases in in vivo aminoacylation by a variety of aminoacyl-tRNA synthetases in *E. coli* (Chattapadhyay et al., 1990; Pallanck & Schulman, 1991, and unpublished results). In the studies reported here, we have shown that substitution of the methionine anticodon CAU with the tryptophan anticodon CCA is in itself sufficient to allow $tRNA^{fMet}$ to be aminoacylated with tryptophan in vivo, emphasizing the important role of the anticodon sequence in substrate recognition by TrpRS. The mutant initiator tRNA retains partial methionine acceptor activity, however, indicating that it lacks some structural feature(s) essential for specific aminoacylation with tryptophan. The ratio of initiation of DHFR synthesis with methionine versus tryptophan is 60:40 (Table I); however, this may not accurately reflect the ratio of methionine to tryptophan aminoacylated to the mutant initiator tRNA. In vitro studies have shown that while other amino acids can substitute for methionine at various steps in the initiation pathway, methionine is more efficiently utilized (Giegé et al., 1973a,b; Sundari et al., 1976; Leon et al., 1979). Thus, tryptophan may be underrepresented in the DHFR product relative to the actual level of attachment of Trp to the tRNA.

Alteration of the discriminator base of $tRNA^{fMet}_{CCA}$ from A73 to G73 resulted in specific aminoacylation of the noncognate tRNA with tryptophan, indicating an important role for this site in tryptophan and/or methionine identity. Earlier in vitro studies by others (Uemura et al., 1982) showed that insertion of G73 has only a small negative effect (3-fold) on the rate of aminoacylation of wild-type $tRNA^{fMet}_{CAU}$ by MetRS, however, suggesting that the major role of G73 is in tRNA recognition by TrpRS, allowing the tryptophan enzyme to outcompete the methionine synthetase for the mutant tRNA containing G73. In support of this, substitution of G73 with any other nucleotide in $tRNA^{Trp}_{UCA}$ greatly diminished its suppressor activity (Table II). Thus, G73 has a strong positive interaction with TrpRS, and/or other bases at position 73 negatively influence tRNA aminoacylation by the tryptophan synthetase. Comparison of the data in Tables I and II shows that the magnitude of the effect of G73 depends on the tRNA context. Initiation of DHFR synthesis with tryptophan by $tRNA^{fMet}_{CCA}$ containing A73 is stimulated 5-fold by substitution of G73, whereas at least 50 times more tryptophan is inserted by $tRNA^{Trp}_{UCA}$ when G73 rather than A73 is present in the tryptophan tRNA. This difference may be related to the unusual structure of the acceptor stem of $tRNA^{fMet}$, which has a C1·A72 mismatch adjacent to position 73, or it may be related to differences in interaction of TrpRS with tRNAs containing the wild-type CCA versus the mutant UCA anticodon. Recent in vivo and in vitro data indicate that the discriminator base plays a major role in selection of cognate tRNAs by a variety of synthetases [reviewed in Pallanck and Schulman (1992)].

In order to investigate the role of individual anticodon bases in tryptophan identity, single base changes were introduced in the anticodon sequence of $tRNA^{fMet}_{CCA}$/G73. Table III indicates the relative amount of tryptophan inserted at the sites of complementary initiation codons in DHFR by each of the mutants. Although some variation was observed in the steady-state levels of the various tRNA anticodon mutants, the data clearly show that all three anticodon bases are required for in vivo aminoacylation of the tRNA by TrpRS.[2] Base changes at C34 and C35 virtually eliminated the initiation activity. Conversion of CCA to CCG reduced the activity only about 2-fold; however, no tryptophan was detected at the N-terminus of the DHFR protein produced. The anticodon of the $tRNA^{fMet}_{CCG}$/G73 derivative contains two out of three of the bases found in the glutamine anticodon CUG and inserts mainly glutamine (Table I). This represents the first example of in vivo mischarging of a noncognate tRNA lacking U35 with glutamine.

Table III: Effect of Anticodon Base Changes on Initiation of DHFR Synthesis with Tryptophan by $tRNA^{fMet}$ Derivatives Containing G73[a]

anticodons related to Trp (CCA)[b]	rel efficiency of Trp insertion
CCA	100
UCA	0–1[c,d]
GCA	0–1[c]
CAA	0[d]
CCG	0[e]

[a] The efficiency of initiation of DHFR synthesis with tryptophan is relative to that observed with $tRNA^{fMet}_{CCA}$/G73. [b] The underlined nucleotides differ from those in the reference anticodon CCA. [c] No sequence information available. The maximum value given assumes 100% insertion of Trp, and the minimum value of 0 assumes no insertion of Trp. [d] Northern analysis indicated that the UCA and CAA mutants were present at only about one-tenth the level of the CCA derivative. [e] No Trp is detected by Edman degradation.

The data show that $tRNA^{fMet}_{UCA}$/G73 is virtually inactive as an initiator tRNA while $tRNA^{Trp}_{UCA}$ (also containing G73) is an efficient opal suppressor. This difference in efficiency might be partially explained by the lower level of expression of the UCA anticodon derivative of $tRNA^{fMet}$; however, the mutant methionine tRNA might also lack some additional structural features needed to enhance the efficiency of its aminoacylation by TrpRS. A number of tRNAs have been found to have such elements near the end of the acceptor stem. We therefore explored the effect of base pair substitutions at the first three positions of the acceptor stem of $tRNA^{Trp}_{UCA}$ (Figure 3A) on its suppressor activity. Changes at these sites produced relatively small decreases in the level of suppressed protein, with the largest effects resulting from substitution of G2·C71 by C2·G71 and G3·C70 by A3·U70 (Table II). No amino acid other than tryptophan was detected at the site of the opal stop codon in DHFR, indicating no apparent loss of specificity of the mutant tRNAs.

Although most in vivo assays of tRNA identity rely on protein sequencing to indirectly determine the amino acid(s) attached to a given tRNA, sequence analysis cannot reliably detect amino acids present at a level below about 5%. This is far above the level of nonspecific amino acids that could be tolerated in normal protein synthesis; thus protein sequencing is a relatively insensitive method for determination of in vivo aminoacylation fidelity. In order to apply a highly stringent test of the complete switch of $tRNA^{fMet}$ identity from methionine to tryptophan, we chose to convert the mutant initiator tRNA into an elongator tRNA species and then to examine its effect on cell growth. We have previously shown that the

[2] Anticodon base changes per se are not sufficient to eliminate the initiator function of $tRNA^{fMet}$ since active derivatives containing base changes in each of the three methionine anticodon bases have previously been isolated (Chattapadhyay et al., 1990; Pallanck & Schulman, 1991). Loss of initiation activity is therefore correlated with loss of aminoacylation activity.

unpaired base at the 5′ terminus of $tRNA^{fMet}$ inhibits ternary complex formation with elongation factor Tu and GTP (Schulman et al., 1974). In addition, formylation of the aminoacylated tRNA normally blocks its participation in polypeptide chain elongation. RajBhandary and co-workers identified the sites in the tRNA required for formylation and have demonstrated that formation of a normal base pair at the 5′ terminus is sufficient to convert the tRNA into an elongator species, both in vitro and in vivo (Seong & RajBhandary, 1987; Seong et al., 1989; Lee et al., 1991; Varshney et al., 1991b). We inserted the A1·U72 base pair found in wild-type $tRNA^{Trp}$ at the first position of the acceptor stem of $tRNA^{fMet}_{CCA}$/G73 to create a mutant tryptophan-inserting elongator tRNA. In addition, a change was made at the third base pair of the stem to the sequence present in $tRNA^{Trp}$ (see Figure 3B). This change was expected to reduce the level of formylation of the aminoacylated tRNA (Lee et al., 1991), allowing more efficient participation of the $tRNA^{fMet}$ derivative in elongation, and possibly to enhance the efficiency of aminoacylation of the mutant with tryptophan (Table II).

Expression of $tRNA^{fMet}_{CCA}$/G73/A1·U72/G3·C70 (Figure 3B) in vivo was found to have no effect on cell growth, consistent with the specific aminoacylation of the tRNA with only tryptophan. In order to examine the efficiency of in vivo aminoacylation by TrpRS, the ability of the mutant tRNA to replace endogenous $tRNA^{Trp}$ in protein synthesis in rapidly growing cells was examined. A previously isolated strain of *E. coli* containing a chromosomal mutation in the sole structural gene for $tRNA^{Trp}$ was used for this experiment (Yanofsky & Soll, 1977). This strain grows normally at 37 °C but is unable to survive at 42 °C due to degradation of the mutant endogenous $tRNA^{Trp}$ at the higher temperature. Complementation of the strain with plasmid-borne wild-type $tRNA^{Trp}$ allows growth. Figure 4 shows that the elongator mutant of $tRNA^{fMet}$ also allows growth of the strain at 42 °C, whereas the initiator $tRNA^{fMet}$ species does not. Measurement of growth curves showed that the rate of growth in the presence of the mutant tRNA is about one-half that observed in the presence of a similar level of $tRNA^{Trp}$. Since the tRNA is fully aminoacylated in vivo, the slower growth is likely to be due to a weaker interaction of the mutant with EF-Tu·GTP (Schulman et al., 1974) or to a small deficiency in some other step in the translation process. Nevertheless, it is clear that the changes shown in Figure 3B have converted $tRNA^{fMet}$ from a methionine-specific initiator tRNA into an efficient tryptophan-specific elongator tRNA, capable of rapid recycling during protein synthesis in exponentially growing cells.

The available data indicate that the anticodon and discriminator base are important structural elements for specific aminoacylation of tRNAs with tryptophan. Base pairs in the first three positions of the acceptor stem do not affect specificity but may contribute to the efficiency of aminoacylation by TrpRS. The magnitude of these effects will have to be assessed in in vitro experiments. The initiator tRNA also shares common sequences with $tRNA^{Trp}$ at several other sites, including positions 4·69, 5·68, and 7·66 in the acceptor stem and 28·42, 29·41, and 30·40 in the anticodon stem. The possible contribution of these sequences to tryptophan identity remains to be investigated.

Added in Proof

After submission of this paper for publication, a report appeared by Himeno et al. (1991) showing that G73 is an important recognition element for TrpRS and that U72 weakly contributes to $tRNA^{Trp}$ recognition in vitro.

Acknowledgments

We thank Charles Yanofsky for providing *E. coli* strain LS874, Tom RajBhandary for communication of results prior to publication, John Abelson and Jennifer Normanly for supplying plasmid pTAC101 from which our DHFR vector was constructed, and Mike Yarus for providing plasmids containing $tRNA^{Trp}$ derivatives and for bacterial strains. Deoxyoligonucleotides were synthesized by a Core Facility of Albert Einstein College of Medicine. Protein sequencing was carried out in the AECOM Laboratory for Macromolecular Analysis.

Registry No. Guanosine, 118-00-3.

References

Ahrweiler, P. M., & Frieden, C. (1988) *J. Bacteriol. 170*, 3301–3304.

Ben-Bassat, A., Bauer, K., Chang, S.-Y., Myambo, K., Boosman, A., & Chang, S. (1987) *J. Bacteriol. 169*, 751–757.

Celis, J. E., Coulondre, C., & Miller, J. H. (1976) *J. Mol. Biol. 104*, 729–734.

Chang, A. C. Y., & Cohen, S. N. (1978) *J. Bacteriol. 134*, 1141–1156.

Chattapadhyay, R., Pelka, H., & Schulman, L. H. (1990) *Biochemistry 29*, 4263–4268.

Crothers, D. M., Seno, T., & Söll, D. G. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3063–3067.

Eisenberg, S. P., & Yarus, M. (1980) *J. Biol. Chem. 255*, 1128–1137.

Eisenberg, S. P., Soll, L., & Yarus, M. (1979) *J. Biol. Chem. 254*, 5562–5566.

Giegé, R., Ebel, J. P., & Clark, B. F. C. (1973a) *FEBS Lett. 30*, 291–295.

Giegé, R., Ebel, J. P., Springer, M., & Grunberg-Manago, M. (1973b) *FEBS Lett. 37*, 166–169.

Himeno, H., Hasegawa, T., Asahara, H., Tamura, K., & Shimizu, M. (1991) *Nucleic Acids Res. 19*, 6379–6382.

Hirel, P.-H., Schmitter, J.-M., Dessen, P., Fayat, G., & Blanquet, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8247–8251.

Knowlton, R. G., Soll, L., & Yarus, M. (1980) *J. Mol. Biol. 139*, 705–720.

Lee, C. P., Seong, B. L., & RajBhandary, U. L. (1991) *J. Biol. Chem. 266*, 18012–18017.

Leon, M., Dondon, J., LaBouesse, J., Grunberg-Manago, M., & Buckingham, R. H. (1979) *Eur. J. Biochem. 98*, 149–154.

Normanly, J., & Abelson, J. (1989) *Annu. Rev. Biochem. 58*, 1029–1049.

Normanly, J., Ogden, R. C., Horvath, S. J., & Abelson, J. (1986) *Nature 321*, 213–219.

Pallanck, L., & Schulman, L. H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3872–3876.

Pallanck, L., & Schulman, L. H. (1992) in *Transfer RNA in Protein Synthesis* (Hatfield, D., Lee, B. J., & Pirtle, R., Eds.) CRC Press, Inc., Boca Raton, FL (in press).

Raftery, L. A., Egan, J. B., Cline, S. W., & Yarus, M. (1984) *J. Bacteriol. 158*, 849–859.

Schimmel, P. (1989) *Biochemistry 28*, 2747–2759.

Schulman, L. H. (1991) *Prog. Nucleic Acid Res. Mol. Biol. 41*, 23–87.

Schulman, L. H., Pelka, H., & Sundari, R. M. (1974) *J. Biol. Chem. 249*, 7102–7110.

Seong, B. L., & RajBhandary, U. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8859–8863.

Seong, B. L., Lee, C.-P., & RajBhandary, U. L. (1989) *J. Biol. Chem. 246*, 6504-6508.
Smith, D., & Yarus, M. (1989) *J. Mol. Biol. 206*, 503-511.
Smyth, D. G., Stein, W. H., & Moore, S. (1963) *J. Biol. Chem. 238*, 227-234.
Soll, L., & Berg, P. (1969) *Nature 223*, 1340-1342.
Sundari, R. M., Stringer, E. A., Schulman, L. H., & Maitra, U. (1976) *J. Biol. Chem. 251*, 3338-3345.
Uemura, H., Imai, M., Ohtsuka, E., Ikehara, M., & Söll, D. (1982) *Nucleic Acids Res. 10*, 6531-6539.
Varshney, U., & RajBhandary, U. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1586-1590.
Varshney, U., Lee, C.-P., & RajBhandary, U. L. (1991a) *J. Biol. Chem. 266*, 24712-24718.
Varshney, U., Lee, C. P., Seong, B. L., & RajBhandary, U. L. (1991b) *J. Biol. Chem. 266*, 18018-18024.
Yanisch-Perron, C., Viera, J., & Messing, J. (1985) *Gene 33*, 103-119.
Yaniv, M., Folk, W., Berg, P., & Soll, L. (1974) *J. Mol. Biol. 86*, 245-260.
Yanofsky, C., & Soll, L. (1977) *J. Mol. Biol. 113*, 663-677.
Yarus, M., Knowlton, R. G., & Soll, L. (1977) in *Nucleic Acid-Protein Recognition* (Vogel, H., Ed.) pp 391-408, Academic Press, New York.

Articles

A Visual Pigment from Chicken That Resembles Rhodopsin: Amino Acid Sequence, Gene Structure, and Functional Expression†,‡

Shu-Zhen Wang,*,§ Ruben Adler,*,§,∥ and Jeremy Nathans*,∥,⊥

The Wilmer Ophthalmological Institute, Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, and Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received October 3, 1991; Revised Manuscript Received December 30, 1991

ABSTRACT: The amino acid sequence of a rhodopsin-like visual pigment from chickens has been determined by isolating and sequencing its gene. The predicted sequence is between 70% and 80% identical to bovine, human, and chicken rhodopsins and between 40% and 50% identical to human blue, green, and red cone pigments, the chicken red cone pigment, and cavefish long-wave cone pigments. The encoded pigment, produced by transfection of cDNA into cultured cells, absorbs maximally at 495 nm as determined from photobleaching difference spectra and reacts at 20 °C with 50 mM hydroxylamine with a half-time of 16 min. These properties, together with a high p*I* predicted from the amino acid sequence, suggest that this cloned gene encodes the chicken green pigment previously identified by biochemical and spectroscopic studies. This sequence defines a new branch of the visual pigment gene family.

Visual pigments are retinal-containing proteins that mediate photoreception. In vertebrates, different members of the visual pigment family reside in the rod and cone photoreceptors, where they mediate vision under conditions of dim and bright illumination, respectively. It is generally accepted that most vertebrates have only a single class of rod photoreceptors and, within them, a single form of visual pigment, rhodopsin. By contrast, multiple cone types exist in most vertebrates, and these differ from one another with respect to the absorbance properties of their pigments. Hue discrimination (color vision) is achieved by a comparison of the relative extents of excitation of the different cone pigments.

Cones and rods also differ in several other important respects. For example, they contain different isoforms of phototransduction proteins, such as transducin and phosphodiesterase subunits (Gillespie & Beavo, 1988; Lerea et al., 1989; Charbonneau et al., 1990; Li et al., 1990). They also differ morphologically: in rods the outer segments form uninterrupted cylinders containing stacks of isolated photopigment-rich disks, whereas in cones the outer segments are tapered and the disks remain connected to the plasma membrane (Rodieck, 1973). The 100-fold lower current response per captured photon exhibited by cones, compared to rods, is likely to derive from these differences.

The high degree of sequence homology between rod and cone visual pigments and phototransduction proteins suggests a model in which new photoreceptor classes evolve by sequentially recruiting duplicated genes encoding photoreceptor proteins. A comparison of visual pigment sequences indicates that the earliest duplication in the vertebrate lineage occurred before the divergence of birds, fish, and mammals. At the amino acid level, for example, chicken and human rhodopsins are 87% identical, and the long-wave-sensitive pigments of chickens, cavefish (*Astyanax fasciatus*), and humans are greater than 73% identical. In contrast, none of the long-wavelength pigments so far sequenced are greater than 48% identical to rhodopsin (Nathans & Hogness, 1984; Nathans et al., 1986; Takao et al., 1988; Kuwata et al., 1990; Tokunaga

† This research was supported by National Eye Institute Grants EY04859 (R.A.) and EY07828 (J.N.), by an unrestricted grant from Research to Prevent Blindness to the Wilmer Ophthalmologic Institute, and by the Howard Hughes Medical Institute.

‡ The genetic sequence in this paper has been submitted to GenBank under Accession Number J05369.

* Correspondence may be addressed to any of the authors.
§ The Wilmer Ophthalmological Institute.
⊥ Howard Hughes Medical Institute, Department of Molecular Biology and Genetics.
∥ Department of Neuroscience.

0006-2960/92/0431-3309$03.00/0