In Vitro Protein Engineering Using Synthetic tRNA^{Ala} with Different Anticodons†

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ABSTRACT: The use of synthetic tRNA for *in vitro* protein engineering was tested in a coupled transcription/translation system prepared from *Escherichia coli*. DNA sequences similar to the natural tRNA$^{Ala/UGC}$ gene from *E. coli* but with different anticodons were synthesized *in vitro*, cloned into a DNA plasmid, and then transcribed *in vitro* with T7 RNA polymerase. The UGC alanine anticodon was changed to CUA corresponding to the UAG stop codon, CCU corresponding to the rarely used AGG arginine codon, and two four-nucleotide anticodons used to suppress stop codons. Bacterial dihydrofolate reductase was the test protein. Its cloned coding sequence was mutagenized at the GUG codon for valine-75 to correspond to the anticodons of the tRNA constructs, and then the plasmids were used to direct the synthesis of dihydrofolate reductase in the coupled transcription/translation system containing the corresponding synthetic tRNA. The results indicate that all four synthetic tRNAs were functionally active in the synthesis of full-length, enzymatically active dihydrofolate reductase protein.

Synthetic tRNAs have been generated in which the anticodon/amino acid relationships do not correspond to those of the universal genetic code. Such tRNAs with AAA anticodons have been used with polyuridylic acid to direct the synthesis of a number of homopolymeric peptides in a cell-free translation system (Picking et al., 1991a,b, 1992) thereby subverting the universal genetic code. These studies suggest that it will be possible to use the same strategy with synthetic tRNAs for *in vitro* engineering of proteins, i.e., to change the primary sequences of proteins during their synthesis in cell-free translation systems. Suppression of nonsense mutations by tRNAs with anticodons complementary to a termination codon occurs naturally in intact cells and has been studied extensively (Normanly & Abelson, 1989). tRNAs with anticodons that are complementary to the termination codons have been used with termination codons in cell-free translation systems to incorporate derivatized standard amino acids and a variety of nonstandard amino acids into peptides and proteins (Ellman et al., 1992; Bain et al., 1991; Mendel et al., 1992).

Here we report *in vitro* engineering of an enzyme using several synthetic tRNAs for which the identity for the aminoacylation/anticodon relation does not occur naturally. A problem encountered has been competition of the synthetic tRNA with the naturally occurring tRNA, even though rarely used codons corresponding to low tRNA abundance were selected. Two types of peptides were produced: one containing the desired amino acid and one containing the amino acid that corresponds to the universal genetic code. A technique is described whereby this problem can be overcome by using a synthetic tRNA which has an anticodon containing four nucleotides with an appropriately constructed mRNA. The proper reading frame is maintained only when the four-nucleotide codon is read by the synthetic tRNA. Reading of only three nucleotides by a natural tRNA results in shifting the reading frame to include a termination codon. The results are compared with those using a suppressor tRNA for the UAG codon, for which competition occurs with termination factors.

Dihydrofolate reductase (DHFR)[1] cloned into a plasmid from the *E. coli fol A* gene was used as the test system. This DHFR is a monomeric cytosolic protein that consists of 160 amino acids, including the N-terminal methionine (Smith & Calvo, 1980). Its chemical, physical, and enzymatic properties are well characterized, and its crystallographic structure is known at a resolution of 1.7 Å (Bolin et al., 1982). The GUG codon for valine at position 75 was chosen as the site for *in vitro* mutagenesis with the substitution of alanine into the modified protein. Garvey and Matthews (1989) studied the characteristics of mutant DHFR with amino acid replacements at this position and found that substitution of alanine for valine had little detectable effect on the physical or enzymatic properties of the enzyme. The synthetic tRNAs used were enzymatically aminoacylated with alanine. The nucleotide sequence of the synthetic tRNA constructs that were used was based on *E. coli* tRNA^{Ala,UGC} (Sprinzl et al., 1989), as reported previously for tRNA^{Ala,AAA} (Picking et al., 1991b).

MATERIALS AND METHODS

Materials

The Sequenase 2.0 kit used for double-stranded DNA sequencing was from United States Biochemicals Corporation (Cleveland, OH). Plasmid pUC18 was from Bethesda Research Laboratories (Gaitherburg, MD). [α-^{35}S]dATPαS, [^{14}C]- or [^{3}H]alanine, and [^{14}C]leucine were from New England Nuclear (Boston, MA). The Klenow fragment of DNA polymerase I, DNase I, and T4 DNA ligase were from Ambion (Austin, TX). Taq I DNA polymerase and restriction endonucleases were obtained from Promega Biotec (Madison, WI), except *Bst*NI which was purchased from New England Biolabs (Beverly, MA). Rifampicin, deoxyribonucleotides (molecular biology grade), and all other biochemicals were from Sigma (St. Louis, MO). Ribonucleotides were obtained

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[1] Abbreviations: bp, base pair; DHFR, dihydrofolate reductase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; UTR, untranslated region; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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Table I

A. Nucleotide Sequences of the Modified Region of DHFR mRNA

sequence[a]	amino acid incorporated at codon 75							
		Val	Thr	Trp	Val	Lys	Ser	Val
1. GUG	Val (wild type)	GUA 72	ACG 73	UGG 74	GUG 75	AAG 76	UCG 77	GUG 78
2. UAG	Ala[b]				UAG			
3. AGG	Arg or Ala[b]				AGG			
4. AGGU	Ala[b] (UAA ochre[c])				AGGU			
5. UAGG	Ala[b] (UAG amber[c])				UAGG			

B. Nucleotide Sequences of Synthetic $tRNA^{Ala}$ Constructs in Plasmid pUC 18

*Hin*dIII T7 promoter tRNA gene

5′ GAG|AAGCTT|TAATACGACTCACTATA|GGGGCTATAGCTCAGCTGGGAGAGCGCCTGC

*Bam*HI

TTAAAACGCAGGAGGTCTGCGGTTCGATCCCGCGTAGCTCCACCA|GGATCC|$GAGA_{112}$ 3′

*Bst*NI

anticodon		tRNA	codon read
CTA	→	CUA, Ala1	UAG
CCT	→	CCU, Ala2	AGG
ACCT	→	ACCU, Ala3	AGGU
CCTA	→	CCUA, Ala4	UAGG

[a] Four oligodeoxynucleotides (lines 2–5, 21 nucleotides long each) were synthesized that differed in the sequence for codon 75 from the wild type (indicated in line 1). [b] Ala incorporated when read by synthetic $tRNA^{Ala}$ with the corresponding anticodon. [c] Termination codons when $tRNA^{Ala}$ with the four-nucleotide anticodon is not used.

from Pharmacia (Piscataway, NJ). NuSieve GTG agarose and Sea Plaque agarose were obtained from FMC (Rockland, ME). *E. coli* K12, strain A19, was kindly provided by Drs. Wittmann and Nierhaus (Berlin, Germany). The coding sequence for *E. coli* DHFR inserted into pSP65 and an MYI plasmid containing the SP6 RNA polymerase were the kind gifts of Dr. A. Spirin and co-workers (Pushchino, Moscow Region, Russia). Oligodeoxyribonucleotides were synthesized on an Applied Biosystems (Foster, CA) 381A DNA synthesizer.

Methods

Construction of pSP65-DHFR.7 Plasmids. pSP65 DHFR (wild type) was digested with *Sac*I and *Hin*dIII, generating a fragment which contained the coding sequence for *E. coli* DHFR and approximately 500 bp in the 3′-untranslated region (UTR). DHFR.7 (a coding sequence of 480 bp and a shortened 3′-UTR of 165 bp) was produced by PCR from the above pSP65 DHFR (wild type) using two oligomers. The 5′-primer covered nucleotides 2926–2943 of SP65 (pSP65 forward primer). A 3′-primer was synthesized to generate a *Hin*dIII restriction site after nucleotide 165 in the 3′-UTR of DHFR mRNA and to remove the last ~350-bp sequence. The major PCR product was a fragment of the expected size. It was digested with *Sac*I/*Hin*dIII and then purified by electrophoresis on a 1% low-melting agarose gel and ligated into plasmid pSP65, which had been treated with the same restriction enzymes. The ligation reaction mixture was incubated overnight at 16 °C with T4 DNA ligase. *E. coli* strain XL1B was transformed using an aliquot of the ligation reaction mixture. Transformed XL1B was incubated on an LB media plate containing 0.05 mg/mL ampicillin and X-gal. Transformants harboring the recombinant plasmid were screened by PCR using the same primers. Both strands of the insert were sequenced using the Sequenase 2.0 kit following the manufacturer's instructions, except that the plasmid was first denatured in 0.1 M NaOH and 1 mM EDTA at 37 °C, precipitated with ethanol, and hybridized to an oligonucleotide primer for 15 min at 37 °C.

Site-Directed Mutagenesis in the DHFR Coding Sequence at Position 75 by PCR. Site-directed mutagenesis was performed by PCR to assemble overlapping oligonucleotides into longer products to construct wholly synthetic DNA sequences (Higuchi, 1989). To replace Val75 GTG by the amber stop codon TAG or the Arg codon AGG, or to insert a base pair, oligomers (only 5′–3′ shown in Table IA) were constructed for nucleotides 266–287 in the DHFR coding sequence. Two other primers complementary to pSP65 bp 85–104 and bp 2926–2943, respectively, were also synthesized. Thus, by PCR, then ligation, and transformation, DHFR-containing plasmids with the desired nucleotide sequence at codon 75 were constructed and were confirmed by DNA sequencing.

tRNA Preparation and Aminoacylation. Plasmid pUC18 containing the synthetic $tRNA^{Ala,AAA}$ had been constructed previously (Picking et al., 1991b) by applying PCR recombinant DNA strategies (Sambrook et al., 1989). To replace the anticodon AAA with CTA (amber) or CCT (Arg) or to insert a base pair in the anticodon loop, similar mutagenic oligonucleotides were synthesized that contained the bases of the $tRNA^{Ala}$ except the nucleotide substitutions at the anticodon position and included the *Bam*H1 and *Bst*NI overlapping sequences plus four extra nucleotides (see Table IB). Four mutant DNA sequences were generated, from which tRNA-Ala1–4 were transcribed. The procedure used for *in vitro* transcription of the tRNAs has been reported previously (Picking et al., 1991a). The resulting synthetic tRNAs were enzymatically aminoacylated.

The reaction mixture for aminoacylation contained 100 mM Tris-HCl (pH 7.5), 15 mM $Mg(OAc)_2$, 10 mM KCl, 3 mM

ATP, 3.75 mM DTE, 30 mM [^{3}H]alanine (10 000 Ci/mol) or [^{14}C]alanine (175 Ci/mol), 5 μM synthetic tRNA, and 600 μg/mL DE-S150 as a source of alanyl-tRNA synthetase. The latter fraction was prepared by passing *E. coli* post-ribosomal supernatant over a DEAE–Sephacell column equilibrated with 20 mM Tris-HCl (pH 7.5), 10 mM $Mg(OAc)_2$, 350 mM NH_4Cl, and 1 mM DTE in order to remove nucleic acids. The aminoacylation reaction was allowed to proceed for 25 min at 37 °C, after which time the pH was lowered by adding NaOAc (pH 5.0) to give a final concentration of 0.2 M. The sample was then extracted with an equal volume of 70% phenol, and the tRNA precipitated with 3 vol of ethanol. The precipitated tRNA was collected by centrifugation, resuspended in 0.1 M NaOAc (pH 5.0), chromatographed over Sephadex G-50 equilibrated with the same buffer, reprecipitated with ethanol, and finally taken up in H_2O. Its concentration was determined by the absorbance at 260 nm.

In Vitro Cell-Free Synthesis of Proteins. The ribosome fraction used for *in vitro* cell-free protein synthesis was isolated from the S30 fraction prepared according to Zubay (1973) from *E. coli* K12 (A19). The S30 fraction was centrifuged at 47 000 rpm for 4 h in a Beckman Ti 50 rotor. The separated ribosomes retain all of the components necessary for *in vitro* coupled transcription/translation (Kudlicki et al., 1992). The ribosome fraction was stored at −80 °C in small aliquots.

In vitro protein synthesis was usually carried out in a total volume of 30 μL. The reaction mixture contained 50 mM Tris–acetate (pH 8.2), 11–14 mM $Mg(OAc)_2$, 36 mM NH_4OAc, 72 mM KOAc, 2% poly(ethylene glycol)-6000, 2 mM DTT, 1 mM ATP, 0.8 mM each of GTP, UTP, and CTP, 0.5 mM cAMP, 25 mM phosphoenol pyruvate, 0.35 μg of pyruvate kinase, 1 μg of folinic acid, 83 μM [^{14}C]leucine (40 Ci/mol), 200 μM of each of the other 19 amino acids, 20 μg of *E. coli* tRNA (Boehringer), 1 μg of rifampicin, 1.2 A_{260} units of the ribosome fraction, about 1 μg of plasmid DNA with the coding sequences under the SP6 promoter, and 0.5 μg of SP6 RNA polymerase. The reaction mixture was incubated for 30 min at 37 °C.

Product Analysis. The amount of ^{14}C-labeled leucine incorporated into trichloroacetic acid-precipitable product was determined by liquid scintillation counting. The size of the synthesized protein was analyzed by SDS–PAGE according to Laemmli (1970), with modifications introduced by Schagger and von Jagow (1987), after the exposure of the gel to Hyperfilm (Amersham) at −80 °C. The dried gel was analyzed by autoradiography.

Quantitation Assay of E. coli DHFR Activity. The enzymatic activity of DHFR was determined (Osborn & Huennekens, 1958) by oxidation of NADPH as indicated by decreased absorbance at 340 nm with modifications introduced by Baccanari et al. (1975). After *in vitro* protein synthesis, 15 μL of the reaction mixture was withdrawn and included in the enzyme reaction mixture containing 100 mM imidazole chloride (pH 7.0), 10 mM β-mercaptoethanol, 75 μM dihydrofolate, and 100 μM NADPH in 1.25 mL. The reaction was carried out for the indicated times at the indicated temperatures, normally 30 °C. One unit of DHFR activity is defined as the amount of enzyme required to reduce 1 μmol of dihydrofolate/min based on a molar extinction coefficient at 340 nm of 12.3×10^3 for NADPH (Hillcoat et al., 1967).

RESULTS

For the studies described here, the coding sequence of the *E. coli fol A* gene for DHFR was mutagenized at the valine-75 GUG codon to either UAG, AGG, AGGU, or UAGG (Table IA). In the universal genetic code, UAG is the amber termination codon. AGG is an arginine codon which is rarely used in *E. coli* and does not occur in the coding sequence of DHFR mRNA. The other two mRNA constructs contained an extra nucleotide to generate an in-frame termination codon. However, in-phase translation to form a full-length DHFR polypeptide could only be achieved with a synthetic tRNA containing the corresponding four-nucleotide anticodon. Results with these latter DHFR mutants are described below in section 3.

Table II: Enzymatic Aminoacylation of Synthetic $tRNA^{Ala}$ Species Containing Modified Anticodons

tRNA	anticodon	acylation level with alanine (% of *E. coli* $tRNA^{Ala,GGC}$)[a]
Ala1	CUA	98.6
Ala2	CCU	102
Ala3	ACCU	99.7
Ala4	CCUA	83

[a] *E. coli* $tRNA^{Ala,GGC}$ typically accepts about 600 pmol of alanine per A_{260} unit under the conditions used.

The *fol A* gene, mutagenized from the wild type to give an mRNA with a shortened 3′-untranslated region, was inserted into pSP65 under the SP6 promoter for coupled transcription/translation in the modified *E. coli* S30 system using SP6 RNA polymerase added to the reaction mixture as described under Methods. Four different synthetic $tRNA^{Ala}$ species were generated to translate these mRNAs. They were formed conveniently from a plasmid containing a DNA sequence for a synthetic $tRNA^{Ala}$ with an AAA anticodon. The sequence had been constructed in such a way that it could be easily modified (Picking et al., 1991a,b, 1992). The nucleotide sequences of the tRNA constructs used are summarized in Table IB. The details of how the AAA anticodon in this synthetic $tRNA^{Ala}$ was replaced by the anticodon required for the DHFR mutants described above are given under Methods. All new synthetic $tRNA^{Ala}$ species were enzymatically aminoacylated (see Methods), and the results are presented in Table II. Generally, aminoacylation of the synthetic tRNAs was comparable to the levels of aminoacylation obtained with natural *E. coli* $tRNA^{Ala}$.

(1) UAG As Codon 75 in DHFR mRNA. In the first set of experiments, $tRNA^{Ala,CUA}$ was tested with the DHFR mRNA containing UAG at codon 75. *In vitro* coupled transcription/translation was carried out in a fractionated *E. coli* system whose features have been described in detail (Kudlicki et al., 1992). This *in vitro* system contains all of the macromolecular components necessary for translation; it was supplemented with plasmid SP65 containing the DHFR mutant coding sequence under the SP6 promoter and SP6 RNA polymerase. Rifampicin was added to the reaction mixture to inhibit transcription by endogenous *E. coli* RNA polymerase. Other components added are listed under Methods. Translation was monitored by incorporation of [^{14}C]leucine into trichloroacetic acid-insoluble polypeptides. An aliquot of the reaction mixture was withdrawn to determine the enzymatic activity of the product formed. The results are presented in Table III. Protein synthesis was relatively low with the mutant plasmid, but increased when the synthetic $tRNA^{Ala,CAU}$ was added. A peptide corresponding to enzymatically inactive truncated DHFR terminated at codon 75 was produced in the absence of the synthetic tRNA, but total leucine incorporation increased and full-length enzymatically active DHFR was formed in the presence of this tRNA, as

Table III: Effect of $tRNA^{Ala,CUA}$ on the Synthesis and Enzymatic Activity of DHFR (amber-75)[a]

tRNA added (pmol)	protein synthesized (pmol of leucine incorporated)	relative enzyme activity
none	31.0	0.0
80	48.6	0.33
120	59.8	0.56
wild type	113.4	1.0

[a] Protein was synthesized from the DHFR (amber-75, Table IA) mutant in pSP65 by coupled transcription/translation as described under Methods. A 15-μL aliquot was withdrawn to determine enzymatic activity. After incubation, [^{14}C]leucine incorporation into protein was determined. Enzymatic activity was tested in reaction mixtures of 1.25 mL total volume under conditions given in Methods. Enzymatic activity was followed over time by the decrease in absorbance at 340 nm associated with the oxidation of NADPH. Units of DHFR activity are defined under Methods. Blank values for enzymatic activity in reaction mixtures without added plasmid were subtracted. Typically, these were about 0.1×10^{-3} units; wild-type (WT) DHFR gave 5.42×10^{-3} units.

described below. The synthetic tRNA was included without being aminoacylated, further indicating that it is aminoacylated in the reaction mixture by the *E. coli* tRNA synthetase. The product formed is expressed as the incorporation of [^{14}C]leucine into protein. The full-length DHFR contains 11 leucines, whereas the truncated form has seven leucines.

It should be noted that the fractionated S30 system used for coupled transcription/translation has very little endogenous DHFR activity. Thus, the activity of the *in vitro* synthesized enzyme can be easily detected. Transcription/translation with the DHFR(UAG-75) plasmid gave nearly no activity over background. Enzymatic activity is gained by including $tRNA^{Ala,CUA}$ (Table III). About one-half the specific activity of wild-type DHFR was obtained. *In vitro* synthesized wild-type DHFR was calculated to have a specific activity of about 58 units/mg of protein; previously we have published data that indicate a specific activity of 40.5 units/mg of protein for DHFR formed in the cell-free translation system (Kudlicki et al., 1992), whereas the specific activity of isolated native DHFR was originally reported to be 19.1 units/mg (Baccanari et al., 1975). The basis for this apparent discrepancy may be in small differences in the assay systems that were used. An imidazole buffer was used here, whereas phosphate was used previously (Kudlicki et al., 1992).

The results presented in Table III were complemented by an analysis of the radioactive product formed on polyacrylamide gels in the presence of SDS followed by autoradiography. The autoradiogram obtained is shown in Figure 1. In the absence of added tRNA, the primary peptide product of $M_r \approx 9000$ apparently represented the truncated form terminated at amino acid 75, whereas M_r = 18 100 full-length DHFR was synthesized in the presence of the suppressor $tRNA^{Ala}$ (Figure 1).

(2) AGG As Codon 75. A DHFR mutant containing AGG at codon 75 was constructed to test the synthetic $tRNA^{Ala,CCU}$ (Table I). The AGG Arg codon is rarely used in *E. coli* (Aota et al., 1988) and does not occur in wild-type DHFR mRNA (Smith & Calvo, 1980). The corresponding natural $tRNA^{Arg}$ gene occurs in a single copy in the *E. coli* chromosome (Komine et al., 1990). However, under the optimized standard reaction conditions used for translation (adding commercially available total *E. coli* tRNA at 20 μg/30 μL of assay and arginine like all amino acids except leucine at 200 μM), full-length DHFR was produced from the mutated coding sequence in the absence of synthetic tRNA (data not shown). The mutagenized DHFR gene was sequenced and shown to contain AGG at nucleotide

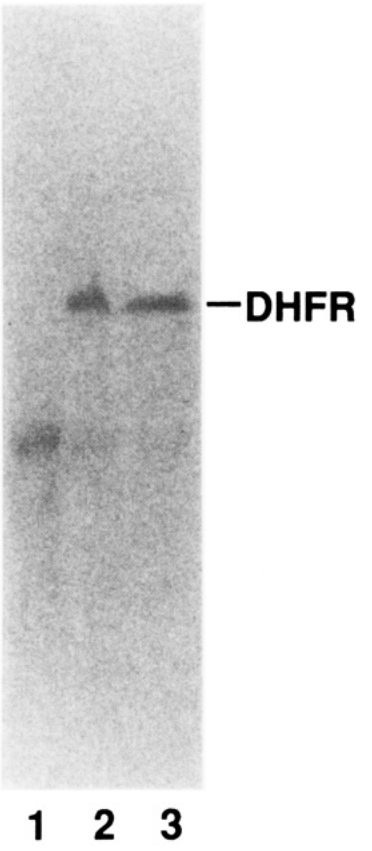

FIGURE 1: Synthesis of full-length DHFR is dependent on suppressor tRNA. The autoradiogram of an SDS–polyacrylamide gel is shown on which the product formed by coupled transcription/translation was analyzed. Lane 1: From a reaction mixture with 1 μg of mutant DHFR (75-amber). Lane 2: From a reaction mixture with 1 μg of mutant DHFR (75-amber) plus 123 pmol of $tRNA^{Ala1}$. Lane 3: From a reaction mixture with 1 μg of plasmid DNA containing the wild-type DHFR sequence.

Table IV: Translation of the DHFR(AGG-75) Gene[a]

Ala-$tRNA^{Ala,CCU}$ added (pmol)	DHFR synthesized (ng)
none	259
55	277
165	317
275	347

[a] Protein was synthesized under conditions similar to those given in the legend to Table III, except that the plasmid and tRNA listed above were used. The weight of DHFR synthesized was calculated from [^{14}C]leucine incorporated into protein using the relation 11 pmol of leucine incorporated = 1 pmol of DHFR = 18.1 ng of DHFR.

residues 223–225 of the coding strand (data not shown). Reduction of the arginine concentration in the protein synthesis reaction mixture to 2 μM caused the amount of product formed to increase in dependence on the amount of Ala-$tRNA^{Ala,CCU}$ that was added to the reaction mixture, as indicated in Table IV.

Replacement of Val75 by Arg destabilizes the DHFR molecules by 2.8 kcal/mol (Garvey & Matthews, 1989). This is reflected in a greater heat sensitivity of the enzyme. To confirm this report, the enzymatic activity of wild-type and mutant Arg75 DHFR was tested over the temperature range from 30 to 55 °C (data not presented). It was found that at 30 °C the specific enzymatic activity (NADH oxidation/min/mg of enzyme protein) was the same for proteins synthesized in reaction mixtures containing the plasmid with the wild-type DHFR gene or those containing the mutant

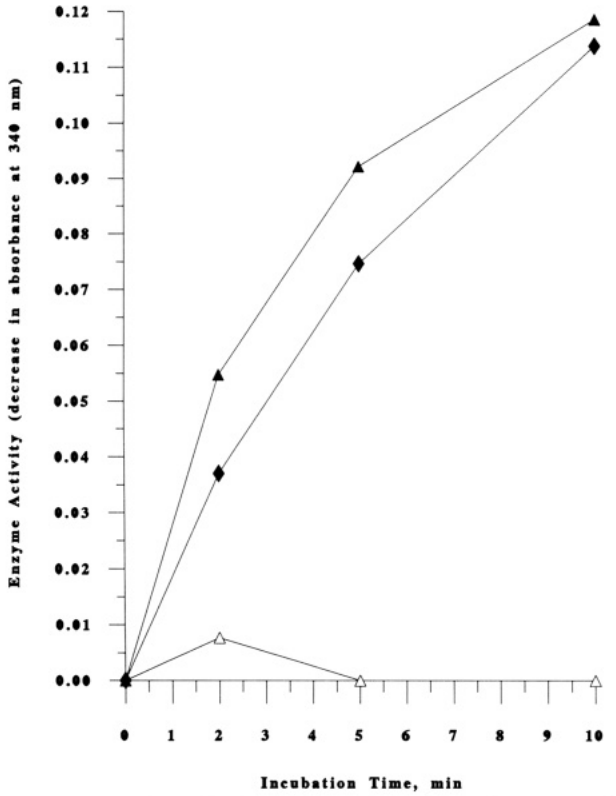

FIGURE 2: Evidence for the incorporation of Ala from synthetic $tRNA^{Ala,CCU}$. The DHFR enzyme assay was carried out at 48 °C with aliquots (15 μL) from the coupled transcription/translation reaction mixtures and other components listed under Methods: △, DHFR synthesized from plasmid (75-AGG), no synthetic tRNA added; ◆, wild-type DHFR; ▲, mutant DHFR (75-AGG) with $tRNA^{Ala2}$, 191 pmol added. The decrease in absorbance at 340 nm measured after incubation at 48 °C for the indicated time is plotted.

DHFR sequence either in the absence or presence of Ala-$tRNA^{Ala,CCU}$ (cf. Table IV). However, differences were observed when the experiment was repeated with an assay temperature of 48 °C, as indicated in Figure 2. At 48 °C no enzymatic activity above background was detected in reaction mixtures to which $tRNA^{Ala,CCU}$ had not been added. Presumably only enzyme proteins containing arginine at position 75 were formed under these conditions. However, DHFR synthesized in the presence of the synthetic tRNA had the same enzymatic activity as an equal amount of the *in vitro* synthesized wild-type DHFR. In these experiments, the amount of enzyme protein formed in the *in vitro* system was quantitated from [^{14}C]leucine incorporation into the DHFR protein. These results indicate that primarily alanine from synthetic Ala-tRNA with the anticodon complementary to the AGG codon was incorporated at position 75 of this mutated DHFR sequence.

(3) Synthetic tRNAs with Four-Nucleotide Anticodons. The synthetic $tRNA^{Ala,CCU}$ was modified to contain an extra nucleotide adjacent to the anticodon. An adenine was inserted either 5′ (to give $tRNA^{Ala,ACCU}$) or 3′ to the anticodon (to give $tRNA^{Ala,CCUA}$). These constructs ($tRNA^{Ala3}$ and $tRNA^{Ala4}$) are shown in Table IB. The corresponding modifications of the DHFR coding sequence were made by inserting a uridine residue adjacent to the AGG codon at position 75 (see Table IA). These mutations generate an in-frame stop codon (UAA ochre or UAG amber) when the mRNA is translated with natural tRNAs with anticodons of three nucleotides. In these cases the nascent peptide should be terminated to yield a truncated peptide of 76 or 75 residues, respectively. However, a full-length DHFR polypeptide should be formed if the coding sequence at this point is translated by a synthetic $tRNA^{Ala}$ with the four-nucleotide anticodon.

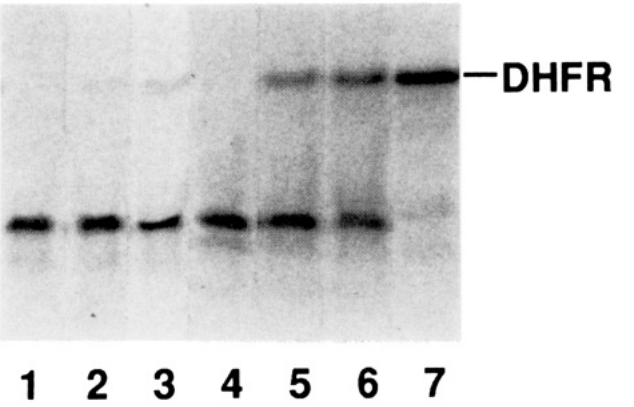

FIGURE 3: Suppression by tRNA with four-nucleotide anticodon requiring higher Mg^{2+} concentrations. An aliquot (15 μL) of the reaction mixture after coupled transcription/translation was analyzed by SDS–PAGE and autoradiography. The autoradiogram is shown. The reaction mixtures were modified in that the specific activity of [^{14}C]leucine was increased to 100 Ci/mol and the Mg^{2+} concentration was changed to give the final concentrations indicated. Lanes 1–3, mutant DHFR (TAGG): lane 1, no tRNA added; lanes 2 and 3, 250 pmol of $tRNA^{Ala4}$. Lanes 4–6, mutant DHFR (AGGT): lane 4, no tRNA added; lanes 5 and 6, 180 pmol of $tRNA^{Ala3}$. Lane 7: Wild-type DHFR. The Mg^{2+} concentration was 12 mM in lanes 1, 2, 4, 5, and 7 and 14 mM in lanes 3 and 6.

Table V: Synthetic tRNA with Four-Nucleotide Anticodon Restores the Biological Activity of DHFR[a]

DHFR	synthetic tRNA added	protein synthesized (pmol of leucine incorporated)	relative enzyme activity
mutant TAGG	–	80	0
	+	111	0.15
mutant AGGT	–	100	0
	+	143	0.4
wild type	–	156	1.0

[a] Protein was synthesized by coupled transcription/translation as described under Methods, except that the Mg^{2+} concentration was raised to 14 mM. Where indicated, the respective tRNA was added (264 pmol for $tRNA^{Ala4}$ and 180 pmol for $tRNA^{Ala3}$, respectively). Enzymatic activity was determined with 15 μL of the *in vitro* protein synthesis reaction mixtures (see legend to Table III).

The results obtained for the ochre mutant in combination with $tRNA^{Ala3}$ and for the amber mutant in the absence or presence of $tRNA^{Ala4}$ are presented in Table V. Both synthetic tRNAs are able to suppress the termination codon generated around position 75 in DHFR by the insertion of a base pair in the gene. Thus, the synthetic tRNA with a four-nucleotide anticodon is active for protein synthesis in the *in vitro* system and generates an enzymatically active enzyme. The results presented in Table V reflect optimal tRNA concentrations and optimal assay conditions. It should be noted that, in these experiments involving the tRNAs with four-nucleotide anticodons, the Mg^{2+} concentration in the reaction mixture was increased to 14 mM. The effect of increasing the Mg^{2+} concentration on the suppression by the tRNAs with the four-nucleotide anticodon is documented in Figures 3 and 4. The product formed was analyzed by SDS–PAGE followed by autoradiography. An autoradiogram obtained from the polyacrylamide gels in these experiments is shown in Figure 3. Nearly no suppression was observed at 11 mM Mg^{2+}; the best results were obtained at 14 mM Mg^{2+}. Further increases in the Mg^{2+} concentration did not produce more full-length DHFR. An autoradiogram similar to the one shown in Figure

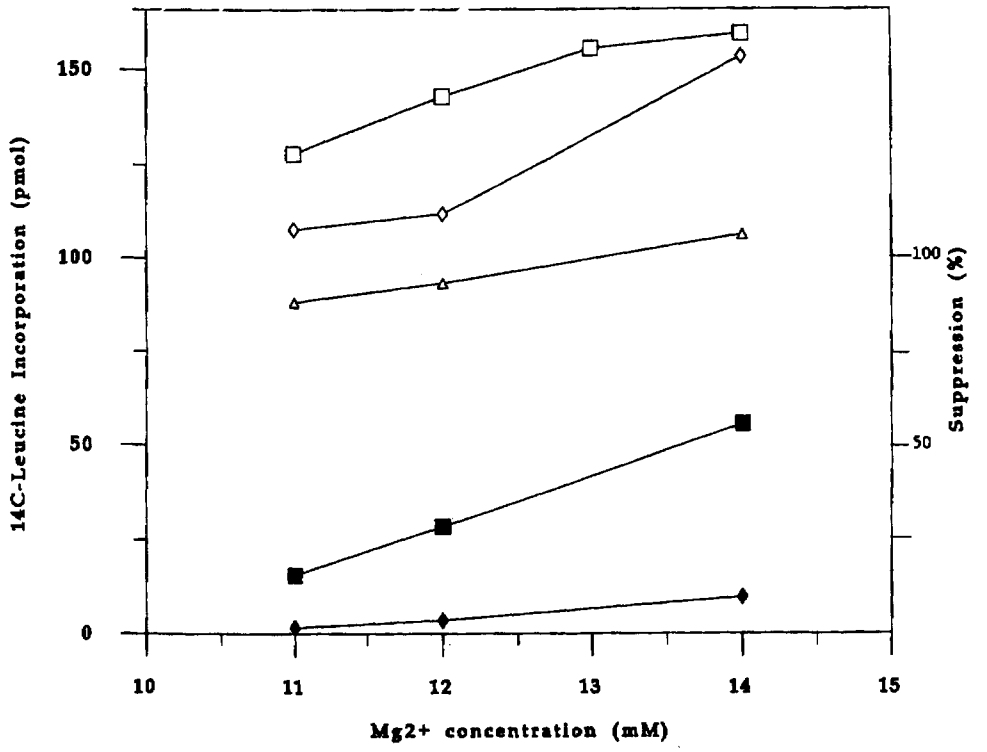

FIGURE 4: Relation between Mg^{2+} concentration and suppression. Reaction mixtures for coupled transcription/translation contained the final Mg^{2+} concentration indicated on the abscissa. The reaction mixtures were set up to contain wild-type or mutant DHFR, the latter with the respective $tRNA^{Ala3}$ or $tRNA^{Ala4}$ in the same concentration as given in the legend to Figure 3. After incubation, an aliquot (15 μL) was analyzed by SDS–PAGE and autoradiography. Another aliquot was used to determine [^{14}C]leucine incorporation into protein. The latter data were converted to picomoles of leucine incorporated per 30 μL of reaction mixture. These results are presented by the open symbols: □, wild type; ◇, mutant DHFR (75-AGGT); △, mutant DHFR (75-TAGG). The autoradiogram was scanned using the NIH Image 1.45 program for Apple computers. The percentage area of the full-length DHFR form was calculated and is presented by the closed symbols: ■, mutant DHFR (75-AGGT); ◆, mutant DHFR (75-TAGG).

3 was scanned using a computer program for quantitative evaluation of the suppressor efficiency. Wild-type or mutant DHFR was synthesized; the latter was synthesized in the presence of optimal concentrations of $tRNA^{Ala3}$ and $tRNA^{Ala4}$, respectively, with increasing concentrations of Mg^{2+}. An aliquot was taken after incubation to determine the incorporation of [^{14}C]leucine; another aliquot was analyzed by SDS–PAGE and autoradiography. The autoradiogram was scanned for the areas of truncated vs full-length DHFR. The results are presented in Figure 4. The data indicate a quantitatively more efficient suppression by the $tRNA^{Ala3}$ that contains the extra nucleotide 5′ of the usual anticodon position. Furthermore, suppression by each tRNA containing the four-nucleotide anticodon is more efficient at the elevated Mg^{2+} concentration. These results complement those given in Table V.

DISCUSSION

The results presented here demonstrate that synthetic tRNAs with identity site–codon relations other than those corresponding to the universal genetic code can be used for protein synthesis in the cell-free translation system. The results suggest that the basic strategy can be used to carry out protein engineering *in vitro*. It appears to be feasible to incorporate a number of amino acids from a single codon at a specific site in a target protein by using a series of synthetic tRNAs with the same anticodon but different identity site, although of course this strategy would be restricted to those aminoacyl-tRNA synthetases that do not include the anticodon as a dominant recognition site on the tRNA (Normanly & Abelson, 1989).

The rationale for the experiments described here is based on reports that the G_3U_{70} base pair in *E. coli* $tRNA^{Ala}$ constitutes the primary identity site for recognition by the alanine-specific tRNA synthetase (Normanly & Abelson, 1989). Thus the anticodon could be changed with very little effect on aminoacylation by an *E. coli* synthetase fraction (Table II). All synthetic $tRNA^{Ala}$ constructs described here were active for *in vitro* protein synthesis. This could be easily demonstrated for those cases where, in the absence of the synthetic tRNA, only truncated DHFR was formed. Efficient suppression was observed (Figure 1), and enzymatically active DHFR was produced (Table III) in the presence of $tRNA^{CUA}$.

Full-length DHFR was synthesized efficiently, even though codon 75 had been mutagenized to AGG. This is a rarely used arginine codon in *E. coli* (Aota et al., 1988). The corresponding $tRNA^{Arg}$ has been classified as a minor species in *E. coli* (Ikemura, 1981), and its gene occurs in a single copy in the *E. coli* chromosome (Komine et al., 1990). Our results indicate that introduction of the single AGG codon in DHFR mRNA did not decrease the amount of protein formed during the 30-min incubation under standard conditions compared to translation of the wild-type DHFR mRNA. However, reduction of the concentration of arginine in the reaction mixture allowed the synthesis of mutant DHFR containing alanine at codon 75 incorporated from the synthetic $tRNA^{Ala\text{-}CCU}$, apparently indicating competition with the naturally occurring $tRNA^{Arg\text{-}CCU}$.

This result reflects one of the problems that we have encountered for *in vitro* protein engineering using the strategy indicated above, even though infrequently used codons were selected that are normally translated by tRNAs with very low abundance. A comparable situation exists for termination

codons, except that of course the competition involves termination factors rather than a natural tRNA species. A system is described with which this problem can be largely circumvented. A novel type of synthetic tRNA is described in this report: tRNA with a four-nucleotide anticodon. Such tRNAs are well-known as frame-shift suppressors in intact cells (Roth, 1981). The data (Table V) indicate that these tRNAs are active for *in vitro* translation. Use of these tRNAs during protein synthesis requires a higher Mg^{2+} concentration. It has been reported that in high Mg^{2+} concentration (50 mM) unmodified tRNA molecules that were synthesized *in vitro* assume a structure more similar to that of native tRNA (Hall et al., 1989). However, enzymatic aminoacylation of the synthetic tRNAs used here was not impaired under standard conditions (10 mM). Thus, we assume that the optimal Mg^{2+} concentration of 14 mM for coupled transcription/translation in the presence of synthetic tRNAs with four-nucleotide anticodons may be necessary for their interaction with the coding sequence.

A positive effect of elevated Mg^{2+} levels on suppression by chemically misacylated iodotyrosyl-$tRNA^{Gly,CUA}$ has been observed when this tRNA was used in the reticulocyte lysate (Bain et al., 1991). It should be noted that the transcription/translation system itself is very sensitive to the Mg^{2+} concentration. Noren and co-workers (1989) optimized the Mg^{2+} concentration used for each preparation of plasmid, suppressor, and S30. We fully concur and suggest that Mg^{2+} concentration should be adjusted to optimize suppression and enzyme synthesis in the specific system that is under study. Incorporation of alanine from $tRNA^{Ala3}$ (ACCU anticodon) appears to be more efficient than from $tRNA^{Ala4}$ (CCUA anticodon). This is probably not due to the type of stop codon, but rather to the position of the stop codon relative to the four-nucleotide codon read by the tRNA; the out-of-phase UAA ochre codon follows the AGGU codon that is read by the synthetic tRNA. The in-phase UAG amber codon precedes the UAGG codon read by $tRNA^{Ala4}$. There is likely to be competition for the termination factor for the latter, whereas in the case of the ochre codon the competition would be from the $tRNA^{Arg,CCU}$. This tRNA is a minor tRNA species which may not compete as successfully with the synthetic tRNA as does the termination factor. An alternative explanation for the difference in incorporation may be that the extra nucleotide 5′ to the anticodon provides for more efficient interaction with the four-base codon than does the extra nucleotide on the 3′-side.

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