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In vitro suppression of an amber mutation by a chemically aminoacylated transfer RNA prepared by runoff transcription

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ABSTRACT

An amber suppressor tRNA was prepared *in vitro* by runoff transcription with T7 RNA polymerase. Both full-length tRNA and truncated tRNA lacking the 3′ terminal pCpA from the acceptor stem could be synthesized from the same DNA template. Truncated runoff suppressor tRNA could be enzymatically ligated to phenylalanyl-pCpA to generate aminoacylated full-length suppressor tRNA (Phe-tRNA$_{CUA}$). Phe-tRNA$_{CUA}$ is capable of suppressing an amber (UAG) mutation *in vitro* with equivalent efficiency as suppressor prepared by anticodon-loop replacement of a naturally-isolated tRNA. The ease of suppressor tRNA preparation using this method, compared to anticodon-loop replacement, greatly facilitates the use of chemically acylated suppressor tRNA's for site-specifically incorporating unnatural amino acids into proteins.

INTRODUCTION

The powerful technique of oligonucleotide-directed mutagenesis is limited, by the nature of the biosynthetic machinery, to substitutions involving the twenty naturally-occurring amino acids. We have recently developed a general method that extends this technique by allowing the site-specific incorporation of unnatural amino acids into proteins (1). The method involves the following steps: conventional oligonucleotide-directed mutagenesis is used to introduce the amber stop codon TAG at the position in the gene corresponding to the amino acid of interest; a suppressor transfer RNA (tRNA) specific for this codon is acylated chemically with the desired unnatural amino acid; the acylated tRNA is added to an *in vitro* transcription/translation system that is programmed with the mutagenized DNA; and the unnatural amino acid is incorporated into the synthesized polypeptide specifically at the position corresponding to the amber mutation.

In addition to efficiently suppressing amber mutations *in vitro*, the suppressor tRNA to be used in this method must be neither acylated nor deacylated by any of the endogenous aminoacyl-tRNA synthetases that are present in the S-30 cell extract (2) (from *E. coli*) that is used for *in vitro* protein biosynthesis. This ensures not only that the aminoacylated suppressor will remain acylated prior to binding in the ribosomal A site, but also that the suppressor will not be reacylated with one of the twenty naturally occurring amino acids after it gives up its unnatural amino acid on the ribosome. If this were not the case, a heterogeneous population of proteins would be synthesized with at least two different amino acids incorporated at the position corresponding to the TAG codon, rendering characterization of the mutant impossible.

In our initial work, a suppressor tRNA derived from phenylalanine-specific tRNA (tRNA^{Phe}) from yeast was used (3). Previously it had been shown that not only will yeast tRNA^{Phe} suppressors efficiently suppress amber mutations *in vitro* (4), but also that yeast tRNA^{Phe} is recognized by the *E. coli* phenylalanyl-tRNA synthetase less than 1% as well as *E. coli* tRNA^{Phe} (5). The anticodon replacement procedure of Bruce and Uhlenbeck (6) was used to convert commercially available yeast tRNA^{Phe} to the amber suppressor tRNA. This procedure proved to be very labor-intensive and low-yielding, requiring numerous chemical and enzymatic steps as well as the full chemical synthesis of a tetraribonucleotide containing the amber anticodon.

The anticodon loop replacement procedure can be avoided outright by generating the suppressor tRNA using the technique of runoff transcription (7). The amber anticodon CUA, as well as a truncated 3′ end that facilitates chemical aminoacylation, can be easily engineered at the level of the DNA template that is used for transcription. We demonstrate here that, despite the lack of post-transcriptional base modifications, a suppressor tRNA synthesized by runoff transcription is capable of suppressing an amber mutation *in vitro* to a level equivalent to that observed with suppressor tRNA constructed by anticodon replacement. Additionally, no proofreading of runoff suppressor by the aminoacyl-tRNA synthetases present in the *in vitro* protein synthesis system was observed.

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MATERIALS AND METHODS

Enzymes and biochemicals

T7 RNA polymerase (8) was isolated from *E. coli* HMS12/pGP1-1/pGP1-5, provided by Prof. Charles Richardson. T4 polynucleotide kinase (9) was isolated from *E. coli* ED8689(λCM21), provided by Prof. Noreen Murray. T4 RNA ligase (10) was isolated from *E. coli* MG583, provided by Prof. Michael Gait. ATP(CTP) nucleotidyltransferase (11) was isolated from *E. coli* UT481/pEC4, provided by Prof. Murray Deutscher. S-30 cell extracts were prepared (12) from *E. coli* strain D-10 (*rna-10, relA1, spoT1, metB1*) (13). All restriction enzymes were obtained from New England Biolabs. T4 DNA ligase was from International Biotechnologies, Inc. Calf intestinal alkaline phosphatase and GTP were from Boehringer-Mannheim. Pyruvate kinase, ATP, CTP, and UTP were from Calbiochem. Human placental ribonuclease inhibitor (RNasin) was from Promega. All other biochemicals were from Sigma. Reagents for automated DNA synthesis were from Cruachem, and oligodeoxyribonucleotides were synthesized using a Biosearch 8600 DNA synthesizer.

Suppressor $tRNA_{CUA}$ was prepared from yeast phenylalanine-specific tRNA (Boehringer-Mannheim) using the anticodon loop replacement procedure of Bruce and Uhlenbeck (6), with modifications described elsewhere (1). The RNA dimer pCpA was synthesized and chemically aminoacylated with phenylalanine as previously described (1). The β-lactamase expression vectors pSG7 and pF66*am* have been previously described (1). Plasmids were rigorously purified by a combination of isopycnic banding and anion exchange HPLC as previously described (1).

Construction of runoff expression vector pYPhe2

Four synthetic oligodeoxyribonucleotides, 5′-CGCTGCAGTA-ATACGACTCACTATAGCGGATTTAGCTCAGTTGGGAG-AGCGCCAG-3′, 5′-TTTAGAGTCTGGCGCTCTCCCAACTG-AGCTAAATCCGCTATAGTGAGTCGTATTACTGCAGCG-GTAC-3′, 5′-ACTCTAAATCTGGAGGTCCTGTGTTCGATC-CACAGAATTCGCACCAGGTGCAGGTA-3′, and 5′-AGCTT-ACCTGCACCTGGTGCGAATTCTGTGGATCGAACACA-GGACCTCCAGA-3′ were purified on 8% denaturing polyacrylamide gels, 5′ phosphorylated (15), and annealed by combining 100 ng of each in 25 μL of TE buffer and incubating at 90° C for 2 min, 65° C for 5 min, and 37° C for 30 min. The annealed construct was ligated to double-stranded M13mp18 RF that had been cleaved with KpnI and HindIII. Single-stranded phage was isolated (16) and the insert was sequenced (17). This initial construct was then inserted into pUC18 for production of large amounts of template (pYPhe1, 2762 bp).

Two synthetic oligodeoxyribonucleotides, 5′-CACCAGGT-GATCCATCCA-3′ and 5′-AGCTTGGATGGATCACCT-3′, were gel purified, and 50 ng of each were annealed at 60° C for 10 min. The duplex was phosphorylated and ligated to gel purified, HindIII and BspMI-cleaved pYPhe1 to generate pYPhe2 (2765 bp). The insert was confirmed by double-stranded DNA sequencing (18).

Digestion of pYPhe2 prior to runoff transcription

Preparative BstNI digests (250 μL) contained 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 150 mM NaCl, 35 μg plasmid DNA, and 100 units of BstNI. Preparative FokI digests contained 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM KCl, 10 mM 2-mercaptoethanol, 35 μg plasmid DNA, and 100 units of FokI. Reactions were incubated for 90 min at 60° C (BstNI) or 37° C (FokI). After phenol extraction, cleaved plasmid was precipitated with ethanol and dried. Cleavage with either enzyme typically resulted in recovery of 30 μg plasmid, which was quantitatively cleaved as indicated by agarose gel electrophoresis.

Runoff transcription of full-length and truncated suppressor tRNA

Transcription reactions contained, in a total volume of 300 μL: 30 μg BstNI or FokI-cleaved pYPhe2, 40 mM Tris-HCl, pH 8.1, 19 mM $MgCl_2$, 5 mM DTT, bovine serum albumin (BSA) to 50 μg/mL, 1 mM spermidine, 4 mM each NTP, 1.2 units of inorganic pyrophosphatase, 240 units of RNasin, and 7800 units of T7 RNA polymerase. After incubation at 42° C for 150 min, reactions were extracted successively with phenol/chloroform and chloroform and precipitated with ethanol. Crude transcript was then precipitated with 3 volumes of ethanol and purified on an 8% denaturing polyacrylamide gel, measuring 42 cm×16 cm×1.5 mm. RNA bands were visualized by staining with 0.02% toluidine blue, cut from the gel, and finely minced. RNA was eluted from the minced gel slice by two succesive treatments with 5 mL of 100 mM sodium acetate, pH 4.5, 1 mM EDTA, 0.1% (w/v) SDS at 37° C for 12 h. Stain was removed by extractions with phenol/chloroform and chloroform, and RNA was precipitated with ethanol. Transcript was then desalted on an FPLC Fast Desalting column (Pharmacia) equilibrated with water, and lyophilized.

Replacement of 5′ triphosphate of transcript with 5′ monophosphate

The dephosphorylation reaction (50 μL final volume) contained the following: 20 μg FokI transcript tRNA, 50 mM Tris-HCl, pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 7 units of calf intestinal alkaline phosphatase. After incubation at 37° C for 1 h, the reaction was extracted once with phenol, three times with phenol/chloroform, and once with chloroform. Dephosphorylated tRNA was precipitated with ethanol, and the pellet was suspended in 50 μL containing 70 mM Tris-HCl, pH 8.1, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, and 30 units of T4 polynucleotide kinase. The reaction was incubated 30 min at 37° C, followed by phenol extraction. RNA was recovered by ethanol precipitation, desalted, and lyophilized. Analytical denaturing polyacrylamide gel electrophoresis indicated quantitative conversion to a species with slightly faster gel mobility than the initial transcript.

Enzymatic reconstruction of truncated acceptor stem of tRNA(-CA)

Acceptor stem reconstruction reactions (30 μL total volume) contained the following: 10 μg $tRNA_{CUA}$(-CA), which had been desalted and lyophilized after gel purification, 20 mM glycine (Na), pH 9.0, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM CTP, 0.5 mM ATP, and 10 ng ATP(CTP) nucleotidyltransferase. After incubation at 37° C for 30 min, the reaction was extracted with phenol. The full-length tRNA was recovered by ethanol precipitation, desalted, and lyophilized.

Chemical aminoacylation of suppressor tRNA(-CA) and *in vitro* protein synthesis

Acylation reactions (80 μl total volume) contained the following: 600 μM pCpA-Phe (40 μg), $tRNA_{CUA}$(-CA) (20 μg, which had been desalted and lyophilized after gel purification), 55 mM

Hepes (Na), pH 7.5, 250 μM ATP, 15 mM $MgCl_2$, BSA at 20 μg/ml, dimethyl sulfoxide (DMSO) at 10 percent (v/v), and 200 units of T4 RNA ligase. After incubation at 37° C for 12 min, 2.5 M sodium acetate, pH 4.5, was added to 10 percent (v/v) and the reaction was immediately extracted with phenol (equilibrated with 0.25 M sodium acetate, pH 4.5), phenol/chloroform, and chloroform. Acylated tRNA was precipitated with ethanol, desalted, and lyophilized. The lyophilized mixture of acylated and nonacylated tRNA was stored at −80° C until immediately prior to its use in *in vitro* protein synthesis reactions.

In vitro protein synthesis reactions (30 μL final volume) contained the following, per mL: 56.4 μmol Tris-acetate, pH 7.4; 1.76 μmol dithiothreitol; 1.22 μmol ATP (Na), pH 7; 0.85 μmol each of GTP (Na), CTP (Na), and UTP (Na), pH 7; 27 μmol potassium phosphoenol pyruvate, pH 7; 0.35 μmol each of the 20 amino acids; 19 mg polyethylene glycol 8000; 35 μg folinic acid; 27 μg pyridoxine·HCl; 27 μg NADP; 27 μg FAD; 11 μg p-aminobenzoic acid; 170 μg *E. coli* tRNA; 36 μmol ammonium acetate; 72 μmol potassium acetate; 9.7 μmol calcium acetate; and 10−14 μmol magnesium acetate (the system is very sensitive to magnesium cation concentration [12], which was optimized for each preparation of plasmid, suppressor, and S-30). Plasmid DNA (100 μg/mL) was preincubated at 37° C for 2 min with the above components, after which suppressor tRNA (167 μg/mL, suspended in a small volume of 1 mM potassium acetate, pH 4.5, immediately prior to use) and S-30 (285 μL/mL) were added. Reactions were incubated at 37° C for 1 hour on a rotary shaker (200 rpm), cooled to 0° C, and centrifuged. The supernatant was then assayed for β-lactamase activity using the chromogenic substrate nitrocefin (19). Two μl of supernatant was added to a pre-equilibrated (37° C) 1 mL cuvette containing 0.1mM nitrocefin in 50 mM phosphate buffer, pH 7, and the change in absorbance at 482 nm was monitored. One nitrocefin hydrolysis unit (1 μmol nitrocefin hydrolyzed/min, ϵ_{482} = 15900 M^{-1} cm^{-1}) corresponds to 0.61 μg enzyme, as quantitated by Bradford assay (Bio-Rad).

RESULTS

Biosynthetic incorporation of unnatural amino acids site-specifically into proteins is accomplished by first mutagenizing the gene of interest such that the codon for the amino acid to be studied is converted to the amber stop codon TAG. A suppressor tRNA is then chemically aminoacylated with the desired unnatural amino acid, and the acylated suppressor is added to a cell-free protein synthesis reaction containing the mutagenized DNA. The biosynthetic machinery then incorporates the unnatural amino acid site-specifically in response to the TAG codon.

We have successfully applied this methodology (1) to substitute three phenylalanine analogs for Phe66 in the hydrolytic enzyme RTEM β-lactamase (20), using a suppressor tRNA constructed from yeast phenylalanine-specific tRNA ($tRNA^{Phe}$) by the anticodon-loop replacement procedure of Bruce and Uhlenbeck (6). The phenylalanine anticodon GAA was removed by first treating the tRNA with weak acid to depurinate the tRNA at the hypermodified purine nucleotide wyosine-37, which is immediately adjacent to the anticodon triplet on the 3′ side. Backbone cleavage with aniline, followed by limited ribonuclease A treatment, removed the anticodon triplet as well as the 3′ terminal CA from the acceptor stem. The RNA tetramer 5′-CpUpApA-3′ was synthesized on millimole scale using

Figure 1. pYPhe2, the DNA template for runoff transcription of both full-length and truncated (−3′ CA) yeast phenylalanine-specific $tRNA_{CUA}$. Cleavage of this template with BstNI (heavy arrows) or FokI (light arrows), followed by runoff transcription, yields full-length $tRNA_{CUA}$ or $tRNA_{CUA}$(-CA), respectively.

standard phosphotriester chemistry (21) and ligated to the 3′ half molecule using T4 RNA ligase. Note that this tetramer contains the amber anticodon CUA as well as adenosine to replace wyosine 37. Adenosine was chosen to satisfy the requirement of a purine at this position for efficient suppression (4). After treatment with T4 polynucleotide kinase, the anticodon loop was resealed with RNA ligase to generate truncated amber suppressor tRNA [$tRNA_{CUA}$(-CA)] in 10% overall yield starting from commercially available yeast $tRNA^{Phe}$. The 3′ truncated tRNA facilitated chemical aminoacylation, since the protected synthetic RNA dimer pCpA could be chemically aminoacylated and ligated to the truncated suppressor with T4 RNA ligase (1). This procedure circumvents the substantial protection problems associated with chemical acylation of a 76-nucleotide tRNA molecule.

We have also generated $tRNA_{CUA}$(-CA) by runoff transcription (7), thereby bypassing the difficult preparation of truncated suppressor by anticodon-loop replacement. The technique of runoff transcription allows the generation of RNA molecules of a defined length from a DNA template using T7 RNA polymerase. The 5′ end is defined by the position of the T7 RNA polymerase promoter (i.e. the penultimate nucleotide of the promoter directs the incorporation of the first nucleotide of the transcript). The 3′ end of the transcript is defined by a cleaved restriction site, with the final nucleotide of the template strand (the 5′ terminus) coding for the 3′ terminal nucleotide of the transcript.

A vector constructed by Sampson and Uhlenbeck (7) for runoff transcription of yeast $tRNA^{Phe}$ was redesigned (Fig. 1) to produce tRNA with the amber anticodon CUA. Cleavage of the original Uhlenbeck construct with BstNI results in template from which full-length tRNA can be transcribed. In order to generate truncated tRNA(-CA) for chemical aminoacylation, a recognition site for the FokI restriction enzyme was introduced downstream from the tRNA gene, positioned such that the enzyme will cleave the template strand between the two guanosines that direct incorporation of C74 and C75 of the tRNA transcript. This way, either an intact acceptor stem or a truncated acceptor stem lacking the 3′ terminal CA could be generated, depending on whether the template is digested with BstNI or Fok1, respectively (Fig. 1). The use of Type IIS restriction enzymes such as FokI, which cleaves a defined number of base pairs upstream from its recognition sequence, allows the generation of a runoff transcript with a 3′ sequence that is independent of the restriction enzyme recognition sequence (22).

The tRNA gene, together with the T7 RNA polymerase promoter, was constructed from six synthetic oligodeoxyribonucleotides ranging from 18 to 67 nucleotides in

length. The construct was inserted into the high copy number plasmid pUC18 for production of large amounts of template. The resulting vector, pYPhe2, was rigorously purified by a combination of isopycnic banding in a cesium chloride gradient and anion exchange HPLC (1) prior to its use in transcription reactions.

Transcription reactions were carried out on 30 μg of BstNI or FokI-cleaved DNA, using highly purified T7 RNA polymerase (8). After gel purification and desalting, 273 μg of the full-length suppressor and 294 μg of the 3′ truncated suppressor were obtained, which represent 660 and 730 transcripts per copy of gene, respectively. Analytical gel analysis (Fig. 2) of the purified transcripts indicated that the full-length (BstNI) suppressor preparation and the truncated (FokI) suppressor preparation were both a 75:25 mixture of the desired product and a product that appears to be one nucleotide longer. Both preparations were used in suppression experiments with no further purification.

Suppression efficiencies were determined by restoration of enzyme activity in *in vitro* protein synthesis reactions programmed with an amber mutant (1). The plasmid pSG7 contains the gene for *E. coli* R-TEM β-lactamase (20) under the control of the strong hybrid tac promoter, with a deletion in the region that codes for the leader sequence that allows for direct expression of active enzyme *in vitro*. The plasmid pF66*am* is identical to pSG7 except that the codon for the conserved Phe66 has been mutagenized to the amber stop codon TAG. Because Phe66 has been demonstrated to be important for enzyme function (1), suppression of an amber mutation at this position with Phe is a necessary condition for generating fully active β-lactamase in *in vitro* reactions primed with pF66*am*.

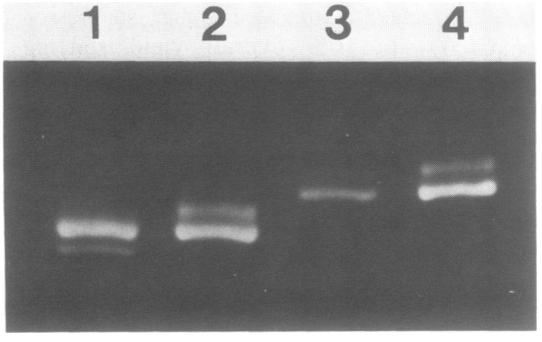

Figure 2. Gel analysis of purified runoff transcripts. An 8% denaturing polyacrylamide gel was loaded with 0.5 μg of each sample and stained with ethidium bromide following electrophoresis. *Lane 1*: $tRNA_{CUA}$(-CA), prepared by anticodon loop replacement of commercially available yeast tRNAPhe. *Lane 2*: $tRNA_{CUA}$(-CA), prepared by runoff transcription using FokI-cleaved pYPhe2. *Lane 3*: Full-length $tRNA_{CUA}$, prepared by ligating pCpA to runoff-transcribed $tRNA_{CUA}$(-CA) with T4 RNA ligase. *Lane 4*: Full-length $tRNA_{CUA}$, prepared by runoff transcription using BstNI-cleaved pYPhe2.

In vitro protein biosynthesis reactions primed with pSG7 yielded 53.8 ± 5.0 nitrocefin (19) hydrolysis units/mL reaction (Table I), which corresponds to 32.8 μg of active enzyme based on a specific activity of 0.61 μg enzyme/nitrocefin unit. No β-lactamase activity was observed in reactions primed with pF66*am* with no suppressor added. Similarly, no activity was observed in pF66*am*-primed reactions that were supplemented with truncated suppressors [$tRNA_{CUA}$(-CA)] prepared either by anticodon-loop replacement or by runoff transcription. Importantly, no activity was observed with full length suppressors prepared from the truncated suppressors either with the *E. coli* repair enzyme ATP(CTP) nucleotidyltransferase (11) or by ligation to pCpA with T4 RNA ligase. Full-length suppressor prepared directly from BstNI-cleaved pYPhe2 also yielded no activity when added to a pF66*am*-primed *in vitro* reaction. These results demonstrate that suppressors synthesized by runoff transcription or by anticodon-loop replacement are not aminoacylated by the endogenous phenylalanyl-tRNA synthetase present in our *in vitro* system.

Truncated suppressors prepared by anticodon-loop replacement or by runoff transcription were aminoacylated by ligation to fully deprotected pCpA-Phe (1) with T4 RNA ligase. The degree of aminoacylation of the runoff transcript was quantitated using dimer aminoacylated with [^{3}H] phenylalanine. The suppressor was acylated to a level of 29 ± 3%, which compares well to the 30−35% level observed using suppressor prepared by anticodon-loop replacement (1). Observed suppression efficiencies (Table I) were 8.6 ± 1.2 nitrocefin units/mL for suppressor prepared by anticodon-loop replacement (16% of wild-

Table I. Comparison of *in vitro* suppression efficiencies of suppressor tRNA prepared by runoff transcription and anticodon loop replacement. Truncated suppressors were prepared by anticodon loop replacement [ALR $tRNA_{CUA}$(-CA)] or by runoff transcription from FokI-cleaved pYPhe2 [FRO $tRNA_{CUA}$(-CA)]. The 3′ acceptor stems of these truncated species were reconstructed with ATP(CTP) nucleotidyltransferase (NTase) or by ligation to either pCpA or phenylalanyl-pCpA (pCpA-Phe). Full-length suppressor was also prepared by runoff transcription from BstNI-cleaved pYPhe2 [BRO $tRNA_{CUA}$]. *In vitro* protein synthesis reactions primed with the indicated plasmids were supplemented with these suppressors. Reactions were incubated and assayed with the β-lactamase substrate nitrocefin as described in Materials and Methods. Data points above background (0.2 nitrocefin hydrolysis units) represent averages of at least 3 experiments.

Plasmid	Suppressor	Acceptor stem extension	Nitrocefin units/mL
pSG7	–	–	53.8 ± 5.0
–	–	–	0.2
pF66*am*	–	–	0.2
pF66*am*	ALR $tRNA_{CUA}$(-CA)	–	0.2
pF66*am*	FRO $tRNA_{CUA}$(-CA)	–	0.2
pF66*am*	ALR $tRNA_{CUA}$(-CA)	pCpA, NTase	0.2
pF66*am*	ALR $tRNA_{CUA}$(-CA)	pCpA, ligase	0.2
pF66*am*	FRO $tRNA_{CUA}$(-CA)	pCpA, NTase	0.2
pF66*am*	FRO $tRNA_{CUA}$(-CA)	pCpA, ligase	0.2
pF66*am*	BRO $tRNA_{CUA}$	–	0.2
pF66*am*	ALR $tRNA_{CUA}$(-CA)	pCpA-Phe, ligase	8.6 ± 1.2
pF66*am*	FRO $tRNA_{CUA}$(-CA)	pCpA-Phe, ligase	8.9 ± 1.3

type) and 8.9 ± 1.3 nitrocefin units/mL for suppressor prepared by runoff transcription (17% of wild-type). The optimum concentration of magnesium cation for efficient suppression was determined to be identical (12 mM) for suppressors prepared by either method.

The effect of a 5′ terminal triphosphate versus a 5′ monophosphate on suppressor function was assayed by removing the 5′ terminal triphosphate from the runoff transcript with alkaline phosphatase, followed by phosphorylation with T4 polynucleotide kinase to afford truncated suppressor with a 5′ terminal monophosphate. Ligation of this suppressor to pCpA-Phe, followed by addition to a pF66*am*-primed *in vitro* reaction, yielded 5.7 nitrocefin hydrolysis units/mL (Table I). This indicates that a 5′ terminal triphosphate does not impair the ability of a runoff-transcribed suppressor tRNA to function on the ribosome. For all intents and purposes, then, suppressor tRNA's prepared by either method display identical behavior in suppressing an amber mutation.

DISCUSSION

We have demonstrated, using a sensitive enzymatic assay for *in vitro* suppression, that suppressor tRNA derived from yeast phenylalanine-specific tRNA, prepared by the method of runoff transcription, suppresses an amber mutation with equal efficiency as suppressor prepared from naturally isolated yeast $tRNA^{Phe}$ by anticodon loop replacement. Both methods yield tRNA with identical sequence, but the *in vitro* runoff transcript presumably lacks the post-transcriptional base modifications that are present in the anticodon-loop replacement product.

A comparison, by Sampson and Uhlenbeck (7), of the aminoacylation kinetics of naturally-isolated yeast $tRNA^{Phe}$ and yeast $tRNA^{Phe}$ prepared by runoff transcription indicated that the runoff transcript, lacking modified bases, is still a good substrate for the yeast phenylalanyl-tRNA synthetase, but with a 5-fold decrease in V_{max}/K_m relative to the naturally-isolated tRNA. The lack of base modification also resulted in an anomalous dependence of aminoacylation on magnesium cation concentration, relative to wild type, with aminoacylation being quite poor at lower magnesium concentrations. The authors postulated that the base modifications stabilize the tertiary structure at low magnesium concentration, but are less necessary at higher magnesium concentrations. The presence or absence of a 5′ triphosphate was found to make no difference with regard to aminoacylation kinetics. In another study (23), it was found that both *E. coli* methionine-specific and valine-specific tRNA's prepared by runoff transcription are also good substrates for their cognate synthetases, with both having an approximate 2.5-fold decrease in V_{max}/K_m relative to the naturally-isolated tRNA's.

The importance of modified nucleotides to translation, rather than aminoacylation, was addressed in a study by Samuelsson *et al.* (24) in which an *M. mycoides* glycine-specific tRNA was synthesized by runoff transcription. Cleavage of a long initial runoff transcript with M1 RNA resulted in $tRNA^{Gly}$ with a 5′ monophosphate. Addition of enzymatically aminoacylated runoff $tRNA^{Gly}$ to a glycyl-tRNA-dependent *in vitro* translation reaction programmed with MS2 RNA yielded synthesized protein at a level of 74% of that observed using enzymatically acylated naturally-isolated tRNA, based on radioactivity incorporation. Recovery and characterization of runoff $tRNA^{Gly}$ following translation indicated the presence of at least one modified base, pseudouridine, that had been introduced by the cell extract used for translation.

Our assay is based on quantitating runoff tRNA efficiency by restoration of enzymatic activity, rather than incorporation of radioactivity. Suppressor tRNA's prepared by anticodon-loop replacement or by runoff transcription display identical suppression efficiencies, based on observed levels of β-lactamase activity. Fourteen of the 76 nucleotides of yeast $tRNA^{Phe}$ are observed to be modified in naturally-isolated tRNA (3). Two of these modified bases, G34 and Y37, are lost during the process of anticodon-loop replacement (6). The remaining twelve modifications do not appear to be important in translation, based on the equivalent suppression efficiency of suppressors prepared by the two methods, although the possibility of the introduction of modified nucleotides by the S-30 extract used for *in vitro* protein synthesis cannot be discounted (24). We have also demonstrated that the presence of a 5′ triphosphate on the tRNA has no observable effect on its function during translation. Additionally, the different magnesium requirements of runoff vs. naturally-isolated tRNA with respect to enzymatic aminoacylation (7) were not observed for translation, since suppressors prepared by both methods display a nearly identical dependence on magnesium concentration in the concentration range studied.

Importantly, the generation of truncated $tRNA_{CUA}$(-CA) using runoff transcription vastly simplifies the unnatural amino acid mutagenesis methodology (1). Runoff transcription reduces the time required for generation of large quantities of suppressor tRNA to days rather than the months required for the anticodon-loop replacement procedure.

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