

Development of improved tRNAs for *in vitro* biosynthesis of proteins containing unnatural amino acids

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Background: Chemically aminoacylated suppressor tRNAs have previously been used *in vitro* to generate mutant proteins in which unnatural amino acids are incorporated site-specifically. Although the existing methodology often provides adequate quantities of mutant proteins, the suppression efficiencies of some unnatural amino acids are not high enough to yield useful amounts of protein. In an effort to make this useful mutagenesis strategy more general, we report here the results of a search to find alternative tRNAs as a way of increasing suppression efficiencies.

Results: Three suppressor tRNAs have been generated by runoff transcription and their ability to deliver unnatural amino acids site-specifically into proteins has been assessed in an *E. coli*-derived *in vitro* transcription/translation system. Analysis of their ability to insert both polar and nonpolar residues in response to an amber codon in two proteins suggests that an *E. coli* tRNA^{Asn}-derived suppressor offers a significant improvement in suppression efficiency over other previously used tRNAs.

Conclusions: Use of an *E. coli* tRNA^{Asn}-derived suppressor may provide substantially higher yields of proteins containing unnatural amino acids, in addition to offering a broader tolerance for polar amino acids. A comparison of suppressor tRNAs derived from tRNA^{Asn}, tRNA^{Gln} or tRNA^{Asp} with that derived from tRNA^{Phe} supports emerging evidence that the identity of an amino acid may be important in message recognition.

Introduction

The ability of tRNAs bearing CUA anticodons to deliver amino acids in response to an amber (UAG) nonsense codon during protein biosynthesis is well documented [1–5]. This ability has been exploited to site-specifically incorporate a wide variety of unnatural amino acids into specific sites in proteins using chemically aminoacylated suppressor tRNAs [6–11] (Fig. 1). A yeast tRNA^{Phe}-derived suppressor [12–15] has been routinely used for this purpose, yet suppression efficiencies for certain (mostly polar) amino acids can be quite low (<15%), making the production of substantial amounts of mutant protein tedious. This has prompted a search for improved suppressor tRNAs to be used in this methodology.

A useful tRNA for the introduction of unnatural amino acids into proteins must satisfy two criteria. First, the suppressor tRNA must not be recognized by any aminoacyl tRNA synthetase present in the *in vitro* transcription–translation system, to avoid enzymatic deacylation or acylation with an undesired amino acid. Second, the acylated tRNA must be readily accepted by the protein biosynthetic machinery. The yeast suppressor tRNA that we currently use meets the first criterion [16], but poor suppression efficiencies of certain amino acids may result from its failure

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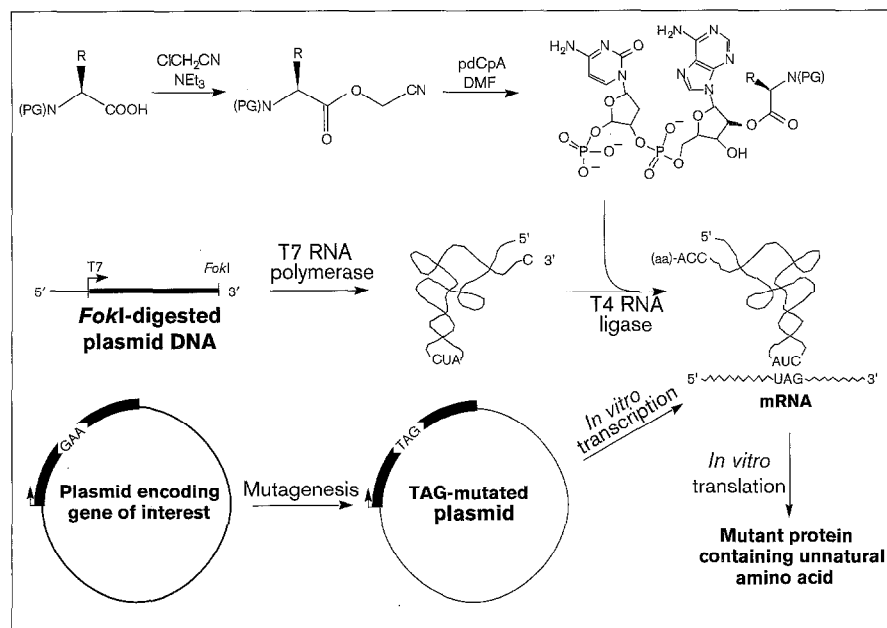
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to uniformly satisfy the latter criterion. In particular, amino acids with highly polar sidechains are generally incorporated in low yield [17]. This observation may be a general result of less favorable interactions of acylated polar amino acids with EF-Tu or the ribosome. On the other hand, yeast tRNA^{Phe} has been shown to bind to the *E. coli* ribosome 1000-fold less tightly than does its *E. coli* tRNA counterpart [18]. This may contribute to reduced suppression efficiencies since the cell extract used in our *in vitro* translation reactions is derived from *E. coli* [19,20]. Consequently, the enhanced affinities of *E. coli* suppressor tRNAs for the ribosome might lead to improved suppression efficiencies for the incorporation of unnatural amino acids into proteins.

More than 20 *E. coli* amber suppressor tRNAs have been constructed and characterized [3–5]. Some function as excellent suppressor tRNAs *in vivo*, affording more than 70% insertion of an amino acid in response to the amber nonsense codon, while others fail to yield detectable levels of suppression. Our development of an improved *in vitro* suppressor tRNA was based on two simple assumptions: (i) tRNAs with anticodon loop mutations that do not act as efficient suppressors *in vivo* are no longer recognized by their cognate aminoacyl-tRNA synthetases, and (ii) these

Figure 1



Unnatural amino acid mutagenesis [8,10]. The cyanomethyl active ester of the unnatural amino acid of interest is coupled chemically to the dinucleotide pdCpA. The resulting acylated dinucleotide is ligated enzymatically to a truncated suppressor tRNA lacking the 3' CA nucleotides. Addition of the full-length acylated tRNA to an *in vitro* transcription-translation reaction containing the TAG-mutated gene of interest affords mutant protein in which the unnatural amino acid has been incorporated site-specifically.

tRNAs, if aminoacylated, would be efficiently accepted by the protein biosynthetic machinery. These assumptions are supported by previous work [5,21] showing that tRNAs with anticodon mutations resulting in poor cognate aminoacyl-tRNA synthetase recognition can efficiently incorporate noncognate amino acids if recognized by noncognate aminoacyl-tRNA synthetases. Based on previous studies of *E. coli* suppressor tRNAs in which tRNA^{Asp}- and tRNA^{Asn}-derived suppressors were found to exhibit poor (<0.1%) *in vivo* suppression efficiencies [4], these tRNAs were deemed promising candidates for the unnatural amino acid mutagenesis methodology. These two tRNAs were generated as described below and their suppression efficiencies were compared with those of the yeast tRNA^{Phe} suppressor [12,22], with a previously characterized *E. coli* tRNA^{Gly}-derived suppressor [11] used in a wheat germ extract system, and with a *Tetrahymena* tRNA^{Gln}(CUA) isoacceptor [23] used in an oocyte expression system. In addition, an opal (UGA) suppressor derived from the *Tetrahymena* tRNA^{Gln} was prepared and tested for *in vitro* suppression. The tRNA^{Asn}-derived suppressor affords high suppression efficiencies for both polar and nonpolar amino acids in two protein systems, expanding the range of amino acids which can be efficiently incorporated using this methodology.

Results

Translation conditions

To examine a range of possible *in vitro* translation conditions, the suppression efficiencies of two different amino acids (valine and homoglutamate) in two proteins (T4 lysozyme and *E. coli* chorismate mutase) were used to evaluate each tRNA. We have previously observed that the

amber codon at 82 of T4 lysozyme is suppressed with good efficiency by the yeast tRNA^{Phe}-derived suppressor, while the amber codon at site 88 in *E. coli* chorismate mutase is suppressed with only modest efficiency. Similarly, nonpolar residues such as valine are known to be inserted by the yeast tRNA^{Phe}-derived suppressor with a consistently high efficiency of more than 50%, while polar residues such as homoglutamate are inserted by this tRNA with a much lower efficiency of typically less than 10% [10].

Suppression of a nonpolar amino acid

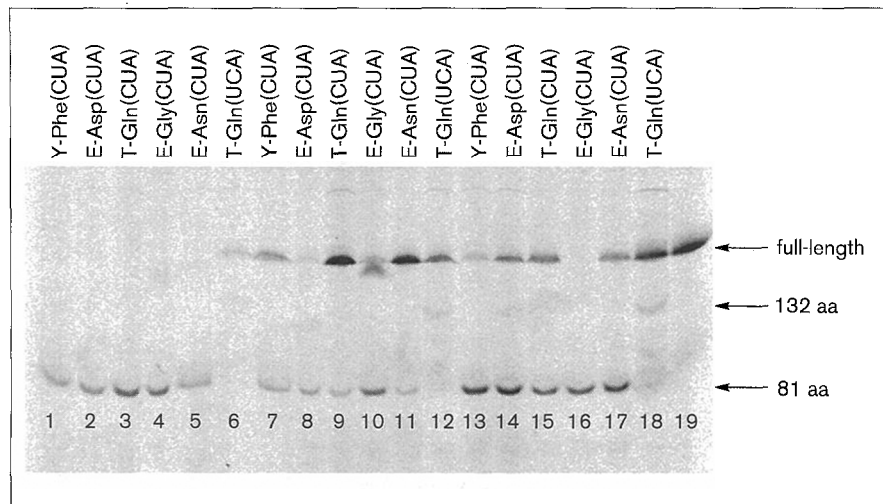
None of the unacylated amber suppressor tRNAs resulted in higher levels of full-length protein (readthrough product) than yeast tRNA^{Phe}(CUA) (Fig. 2). The *E. coli* tRNA^{Asn}(CUA) provided the highest average suppression efficiencies of valine in both T4 lysozyme at site 82 and in *E. coli* chorismate mutase at site 88 of 77% and 75%, respectively (Figs 3,4). These efficiencies represent a significant improvement over those of yeast tRNA^{Phe}(CUA), which afforded 54% and 50% suppression efficiency in T4 lysozyme and in chorismate mutase, respectively, and represent a smaller improvement over suppression efficiencies of the *Tetrahymena* tRNA^{Gln}-derived amber suppressor. The *E. coli* tRNA^{Asp}(CUA) and tRNA^{Gly}(CUA) conferred poorer suppression efficiencies for valine in both proteins (Figs 3,4).

Suppression of a polar amino acid

Whereas the yeast tRNA^{Phe}-derived amber suppressor afforded suppression efficiencies for homoglutamate of <10% in both T4 lysozyme and chorismate mutase, *E. coli* tRNA^{Asn}(CUA) inserted homoglutamate into these proteins

Figure 2

Nonsense suppression in T4 lysozyme. *In vitro* transcription and translation reactions were conducted as described previously [8] in the presence of ^{35}S -Met. A 5- μl aliquot of each 30- μl reaction was subjected to SDS-PAGE (15% acrylamide) and quantitation of the radiolabeled protein was performed with a Molecular Dynamics 445SI PhosphorImager. Lanes 1–6, with unacylated tRNA; lanes 7–12, with valine-acylated tRNA; lanes 13–18, with homoglutamate-acylated tRNA. All reactions used a T4 lysozyme gene containing a TAG codon at residue 82, with the exception of lanes 6, 12 and 18, which used a T4 lysozyme gene bearing a TGA codon at residue 133, and lane 19, which used wild-type T4 lysozyme DNA. The identity of the tRNA is identified at the top of each lane: Y = yeast; E = *E. coli*; T = *Tetrahymena*.



with 34% and 20% efficiencies, respectively. Suppression efficiencies with the *Tetrahymena* tRNA^{Gln}(CUA) were comparable, at 25% and 15%, respectively. Interestingly, *E. coli* tRNA^{Asp}(CUA) incorporated homoglutamate with good efficiency into both proteins (21% and 35%, respectively), despite comparatively poor suppression of valine (Figs 3,4).

Opal codon suppression

Successful suppression of an opal codon with a suppressor tRNA may provide a route to the production of a

protein containing two different unnatural amino acids. The *Tetrahymena* tRNA^{Gln}-derived opal suppressor was generated and aminoacylated as described above. The unacylated tRNA, however, yielded extremely high (>90%) levels of readthrough at site 133 in T4 lysozyme and therefore suppression with the aminoacylated tRNA is likely to result in the insertion of an unknown mixture of amino acids (Fig. 2). Although this high degree of readthrough is consistent with previous studies [24] that have found differences in context-dependent suppression between

Figure 3

Comparison of suppression efficiencies of five tRNAs in T4 lysozyme at site 82. Suppression efficiencies are defined as the amount of full-length protein divided by the sum of the full-length and truncated protein produced in each reaction. The suppression efficiencies shown represent the average of two trials. The tRNAs are identified below each bar: Y = yeast; E = *E. coli*; T = *Tetrahymena*; rt = readthrough (unacylated tRNA); V = acylated with valine; hE = acylated with homoglutamate.

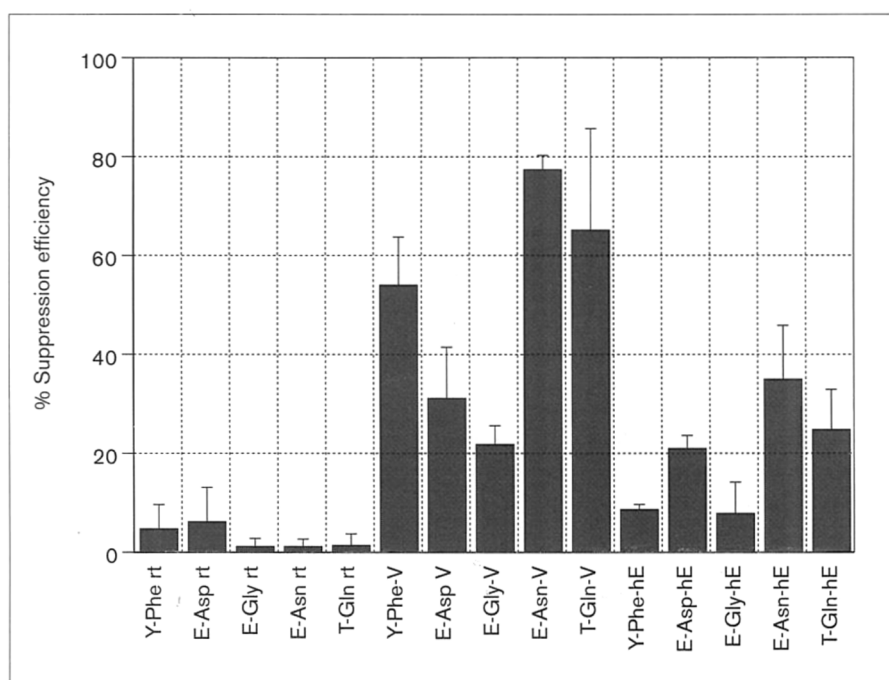
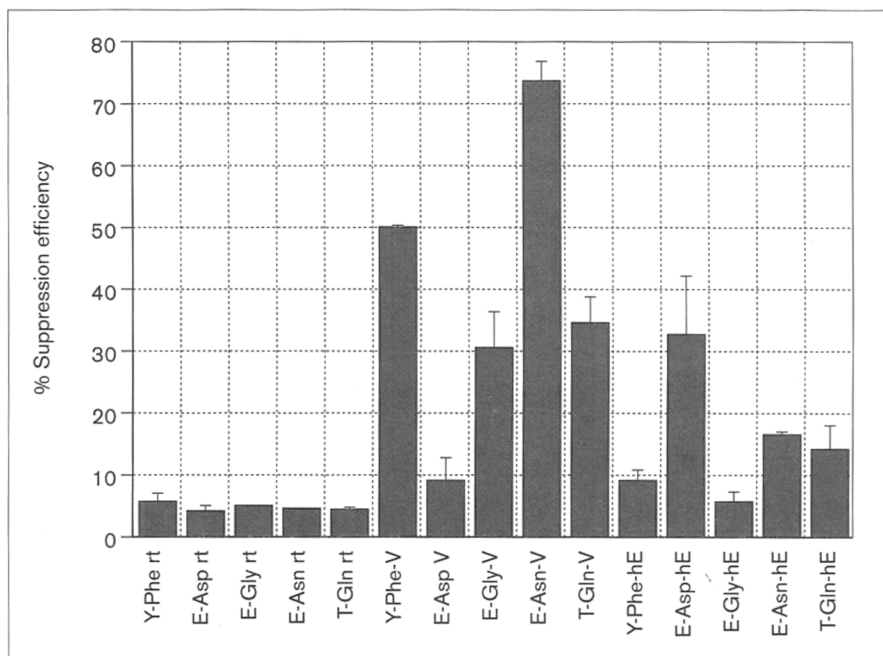


Figure 4



Comparison of suppression efficiencies of five tRNAs in chorismate mutase at site 88. Suppression efficiencies are defined as the amount of full-length protein divided by the sum of the full-length and truncated protein produced in each reaction. The suppression efficiencies shown represent the average of two trials. The tRNAs are identified below each bar: Y = yeast, E = *E. coli*; T = *Tetrahymena*; rt = readthrough (unacylated tRNA); V = acylated with valine; hE = acylated with homoglutamate.

TGA and TAG nonsense codons, we have seen in the past that the context effects observed *in vivo* are often not consistent with those found *in vitro*. The context effects we have observed *in vitro* are complex and thus far suggest no simple correlations. It is also possible that this result may arise from misacylation of the opal suppressor by an endogenous tRNA aminoacyl synthetase.

Discussion

Based on the assumption that mutation of the anticodon of *E. coli* tRNA^{Asp} and tRNA^{Asn} to CUA results in the loss of recognition of these tRNAs by their cognate aminoacyl synthetases, we have examined the utility of these tRNAs for *in vitro* suppression of unnatural amino acids into proteins. Low levels of readthrough and good suppression efficiencies for both valine and homoglutamate make the tRNA^{Asn}-derived amber suppressor an attractive replacement for the yeast tRNA^{Phe}-derived suppressor in *E. coli*-derived translation systems. Efficient incorporation of homoglutamate suggests that the tRNA^{Asp}-derived amber suppressor may function well when inserting polar amino acids. Together, these tRNAs should significantly enhance our ability to generate substantial quantities of protein containing a wide variety of unnatural amino acids. An opal suppressor derived from the *Tetrahymena* tRNA^{Gln} was found to give high levels of readthrough product, eliminating the possibility of using this tRNA to efficiently deliver a second unnatural amino acid site-specifically into proteins.

This work also supports existing evidence that the acceptance of an aminoacylated tRNA by one or more

components of the protein biosynthetic machinery may depend in part on the match between the tRNA and its covalently bound amino acid. It has been previously observed that the EF-Tu in both *E. coli* [25] and mammalian [26] systems can discriminate between methionine- and *N*-formyl-methionine-charged tRNA^{Met}. Indeed, the poorer suppression efficiencies of polar amino acids in the case of the yeast tRNA^{Phe}-derived suppressor may be attributable to discrimination by EF-Tu. Here we show more generally that suppression efficiencies of identical amino acids attached to different tRNAs differ depending on the relationship between the nature of the amino acid and the tRNA. For example, the general trend observed with the yeast tRNA^{Phe}-derived suppressor that nonpolar residues are incorporated more efficiently than charged amino acids [10] is reversed with the tRNA^{Asp}-derived suppressor for suppression at site 88 of chorismate mutase. In addition, the ratio of incorporation of homoglutamate relative to valine was higher in both proteins with tRNA^{Asp}-, tRNA^{Asn}-, or tRNA^{Gln}-derived suppressors than with tRNA^{Phe}- or tRNA^{Gly}-derived suppressors. Discrimination by EF-Tu or other translation factors may explain interesting observations in this work. Thus the amino acid may indeed have a more significant role than that suggested by the original 'adaptor hypothesis' [27], which postulated that message recognition is determined exclusively by the tRNA and not by the attached amino acid. Our continuing use of these new tRNAs to suppress a larger variety of unnatural amino acids at additional sites will provide important data towards the evaluation of this hypothesis.

Significance

Guided by simple design strategies and the previously reported properties of suppressor tRNAs, this study has found several attractive replacements for the yeast tRNA^{Phe}-derived suppressor previously used *in vitro* to deliver unnatural amino acids site-specifically into proteins. In particular, the *E. coli* tRNA^{Asn}-derived suppressor satisfied all the criteria of an improved tRNA for this method, combining low levels of readthrough with high suppression efficiencies for both polar and non-polar residues. By increasing both the yields of mutant proteins and the range of amino acids capable of being incorporated with this methodology, these new tRNAs promise to significantly enhance the power of unnatural amino acid mutagenesis. This study also contributes to the hypothesis that amino acid identity may be a more important role in message recognition during translation than previously thought.

Materials and methods

Constructs for runoff transcription

All restriction enzymes were purchased from New England Biolabs. *E. coli* tRNA^{Asp}(CUA), tRNA^{Asn}(CUA), and *Tetrahymena* tRNA^{Gln}(UCA) were generated from two overlapping synthetic oligonucleotides encoding the tRNA sequence [4] and the T7 RNA polymerase promoter [22]. The 5' nucleotide (nt) of each tRNA was changed, if necessary, to G for optimal transcription; in these cases nt 72 was also changed to C to restore acceptor stem complementarity. The two annealed oligonucleotides were filled in with the Klenow fragment of *E. coli* DNA polymerase I and inserted between the *Kpn*I and *Hind*III sites of plasmid pYPhe2 [22] to give plasmids pEAsp, pEAsn, and pTGlnOp. The sequences of all constructs were verified by dideoxy DNA sequencing [28]. The resulting purified plasmid DNA was digested to completion with *Fok*I to provide template for the transcription of tRNA lacking the 3' terminal CA dinucleotide. The modified *E. coli* tRNA^{Gly}(CUA) [11] was prepared similarly. The plasmid pTHG encoding the *Tetrahymena* tRNA^{Gln}(CUA) [23] was a generous gift from Professor Dennis Dougherty (California Institute of Technology). The sequences of the *E. coli* tRNA^{Asp}- and tRNA^{Asn}-derived suppressors [4] are as follows:

tRNA^{Asp}(CUA): 5'-GGAGCGGTAGTTCAGTCGGTTAGAATACCTGCCTCTAACGCAGGGGGTCGCGGGTTCGAGTCCCCTCCGTTCCGCCA-3'

tRNA^{Asn}(CUA): 5'-GTCCTCTGTAGTTCAGTCGGTAGAACGGCGGACTCTAAATCCGTATGCTACTGGTTCGAGTCCAGTCAGAGGC-CGCCA-3'

Purification of T7 RNA polymerase

T7 RNA polymerase was purified as previously described [8] from *E. coli* BL21(pAR1219) [29] with the following changes: (1) cell lysis was carried out as described except that sonication of the viscous crude extract formed after addition of deoxycholate was reduced to three 10–15 s cycles (100% duty) at ~75% output (350 W) using a 0.5-in horn tip (Virtis Virsonic 475 Cell Disrupter). In lieu of sonication, MgCl₂ was added to the crude cell extract to a final concentration of 10 mM and RNase-free DNase I (Sigma) was added to a final concentration of 50 μg ml⁻¹. The crude cell extract was then incubated at 4°C for ~30 min with mild agitation. (2) An ammonium sulfate fractionation step was added. A saturated solution of (NH₄)₂SO₄ (4°C) was added to the supernatant collected after addition of Polymin P (Sigma) to make a 35% saturated solution. After 15 min, the solution was centrifuged at 39000 × g for 15 min. The polymerase was precipitated by addition of saturated (NH₄)₂SO₄ solution to the supernatant to make a 55%

saturated solution at 4°C. After a 15 min incubation on ice and centrifugation, the protein pellet was resuspended in ~10 ml of buffer C [8] plus 50 mM NaCl. (3) The concentrated crude polymerase was applied to a Sephadex G-25 column (24×200 mm) equilibrated in buffer C plus 50 mM NaCl and eluted at a linear flow rate of 5 cm h⁻¹. Precipitation of protein will occasionally occur during this desalting step; SDS-PAGE analysis of the precipitant shows that T7 RNA polymerase is not a significant component of the precipitated protein. The void volume fractions containing protein were pooled, diluted with an equal volume of equilibration buffer, and directly applied to a S-Sepharose FF column (15×170 mm) (Pharmacia) as previously described. (4) Application of the protein sample and subsequent wash (buffer C plus 50 mM NaCl) of the S-Sepharose FF column were done at a linear flow rate of 65–70 cm h⁻¹. The column was washed with equilibration buffer until the A₂₈₀ of the eluant dropped below 0.05 AU. T7 RNA polymerase was eluted with buffer C plus 200 mM NaCl at a linear flow rate of 9 cm h⁻¹. (5) Purified T7 RNA polymerase eluting from the S-Sepharose FF column was routinely pooled based on A₂₈₀ with 35% (of peak absorbance) cuts on the leading and trailing edge of the elution profile. (6) The purified polymerase was dialyzed (50 kDa MWCO membrane, Spectra/Pro) against four 0.5-l changes of buffer C plus 100 mM NaCl and 50% glycerol over a total of ~8 h. The T7 RNA polymerase was aliquoted and flash-frozen in liquid nitrogen before being placed into long-term storage at –80°C.

Production of tRNA

The *Fok*I-digested templates were extracted twice with 1:1 phenol:chloroform, ethanol precipitated, and subjected to runoff transcription using T7 RNA polymerase. *In vitro* transcription reactions were assembled at room temperature and contained 40 mM Tris-HCl, pH 8.1, 5 mM DTT, 50 μg ml⁻¹ BSA, 10 mM spermidine, 15 mM MgCl₂, 5.75 mM each NTP, 0.1 mg ml⁻¹ *Fok*I digested plasmid template, 0.75 U ml⁻¹ pyrophosphatase (New England Biolabs), 0.5 U μl⁻¹ RNasin (Promega @ 40 U μl⁻¹), and 80 μg ml⁻¹ T7 RNA polymerase. Reactions were incubated at 37°C for 3 h and then extracted with an equal volume of phenol:chloroform, and ethanol precipitated. The pellet was dried, resuspended in TE, and applied to a NAP-5 desalting column (Pharmacia) to remove NTPs. The purity of the final tRNA product was verified by 10% PAGE in the presence of 7 M urea, and was judged by ethidium bromide visualization to be ≥90% for all tRNAs excluding the tRNA^{Asn}-derived suppressor, which was ≥80%. In all cases, the major impurity was a transcription product one base longer than the desired tRNA; deviation from the reaction conditions described above resulted in unacceptably high yields of run-on products. Valine or homoglutamate, N-protected as the 4-nitroveratrylcarbamates, were acylated onto pdCpA as previously described [8] and the truncated tRNAs were ligated to these aminoacylated dinucleotides using T4 RNA ligase [8]. Unacylated pdCpA was also ligated to the truncated tRNAs to afford full-length unacylated tRNA for use as controls to measure suppression due to enzymatic acylation. The ethanol precipitated and washed ligation products were dissolved in 1 mM potassium acetate (5 μl per 10 μg tRNA), photodeprotected for 30 min at 25°C using a 450 W mercury immersion lamp with a quartz filter, and added directly to the *in vitro* transcription and translation reactions [8].

Measurement of suppression efficiency

In vitro transcription and translation reactions with amber suppressor tRNAs were performed as described previously [8], using plasmids encoding either T4 lysozyme bearing a TAG codon at residue 82 [30] or *E. coli* monofunctional chorismate mutase 25 with a TAG codon at site 88. Opal suppressions using the *Tetrahymena* tRNA^{Gln}-derived opal suppressor were performed using T4 lysozyme plasmid DNA bearing a TGA codon at residue 133. The final concentration of divalent magnesium was 3 mM in the T4 lysozyme reactions and 7 mM in the chorismate mutase reactions. The resulting ³⁵S-Met-labeled crude reaction mixtures were subjected to SDS-PAGE and the amounts of truncated and full-length proteins were quantified using a Molecular Dynamics 4455I PhosphorImager. Suppression efficiencies were defined as the amount of full-length protein divided by the sum of the full-length and truncated products.

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