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Two limitations inherent in phage display are the relatively small library size (less than $10^9$) and the constraint that the building blocks of the library be restricted to the 20 naturally-occurring amino acids. These constraints likely prevented us from identifying ligands for the MUC-1 target form two different phage display libraries, and we have therefore been compelled to seek an alternative method for the presentation of larger and more diverse libraries of potential ligands to the MUC-1 target. The purified translation system we have developed has the potential to overcome both restrictions encumbering phage display because of a much larger library size and an expanded range of unnatural amino acid building blocks beyond the 20 naturally-occurring amino acids. We have now demonstrated that our purified translation system is capable of synthesizing peptidomimetics with unnatural amino acids encoded at sequential positions of the oligomer. A library of such peptidomimietics should overcome the inherent limitations of mirror image display that requires chemically synthesized small targes.

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Appendix 1. Forster et al. manuscript (5 pp total)

## INTRODUCTION:

The ultimate goal of these experiments is to identify degradataion-resistant ligands that bind to a breast cancer-specific epitope of the DF3 protein product of the MUC-1 gene (Siddiqui et al., 1988). The proposed approach was to identify from a phage display library L-peptides that bind to the mirror image D-form of the native ligand (Schumacher et al., 1996). However, mirror image phage display failed to identify any peptides binding to the MUC-1 target.

Current work is focused on overcoming the inherent limitations of phage display libraries. Thus, we are developing a technology that we term "purified ribosome display" for the purpose of selecting peptidomimetics composed of a sequence of unnatural amino acids that bind to the MUC-1 target.

## BODY:

*Task 1.* To select a D-peptide ligand that binds to the breast cancer-specific epitope of the DF3 glycoprotein product of the MUC-1 gene (months 1-18)

- Synthesize and purify discrete oligomers of the 20-residue epitope from the MUC-1 gene product, starting with monomeric building blocks of the D enantiomer (months 1-6)

As related in the 2001 annual report, an oligomeric form of the 20 amino acid MUC-1 target sequence, containing three tandem copies of the repeat and spanning 60 amino acids, was synthesized commercially by QVC biochemicals. The D-peptide contains a biotin group coupled to the N-terminus.

- Optimize conditions for selection of peptide ligands from a phage display library (months 6-12) and identify a consensus sequence for putative candidate peptide ligands (months 6-18).

As related in the 2001 annual report, we screened both a commercial random hexapeptide library, and a second library constructed by Dr. Ton Schumacher (Schumacher et al., 1996) with a 10 residue random sequence flanked by ser/cys at either end using phage display. Biopanning for phage binding to both the S-protein control and the MUC-1 target was performed using both acid and peptide-based elution strategies. Although phage with clear consensus sequences were identified for the control S-protein target, mirror-image phage display failed to identify a peptide that binds to the D-MUC-1 target amino-acid sequence.

The failure to identify a consensus binding sequence for the MUC-1 target by mirror image phage display is likely due to three factors. First, the MUC-1 target polypeptide is inherently flexible and relatively unstructured (Fontenot et al., 1995). As a result, MUC-1 may be a particularly difficult target because both the target (MUC-1) and the ligand (selected peptide) must pay an entropic penalty upon formation of complex (due to the conformational restriction that would be required in both MUC-1 and peptide ligand to enable molecular recognition). Second, the size of phage display libraries is limited by transformation efficiencies to about $10^8$ molecules, several orders of magnitude less than the library sizes that are achievable by other methods, such as ribosome display (Mattheakis et al., 1994). Third, the molecular diversity of the phage-display library is limited by the nature of the side-chains of the 20 natural amino acids.

In the first report period, we developed a purified translation system for synthesis of oligopeptides (Forster et al., 2001), and in the 2002 annual report, we indicated that our top priority is to develop a technology that we term "purified ribosome display." This approach, which will allow us to achieve a library size of up to $10^{15}$ peptides, would dramatically improve upon the existing system for polysome display, which uses crude cell lysates and is therefore both technically challenging and subject to reproducibility problems (Mattheakis et al., 1994). More importantly, such a purified translation system for ribosome display will enable us to increase the diversity of the library further by specifically incorporating unnatural amino acids into the library. If such peptidomimetic libraries could be synthesized from protease-resistant amino acid analogs (e.g. D-amino acids or N-methyl amino acids), then it would be possible to directly select protease-resistant ligands using natural target molecules ("peptidomimetic display"). This would overcome the inherent limitation of mirror image display that requires chemically synthesized small targets.

In May 2003, we published a manuscript in the Proceedings of the National Academy of Sciences demonstrating that our purified translation system is capable of synthesizing peptidomimetics with unnatural amino acids encoded at sequential positions of the oligomer (Forster et al., 2003). The key findings reported in this manuscript (see also Appendix 1) are summarized below.

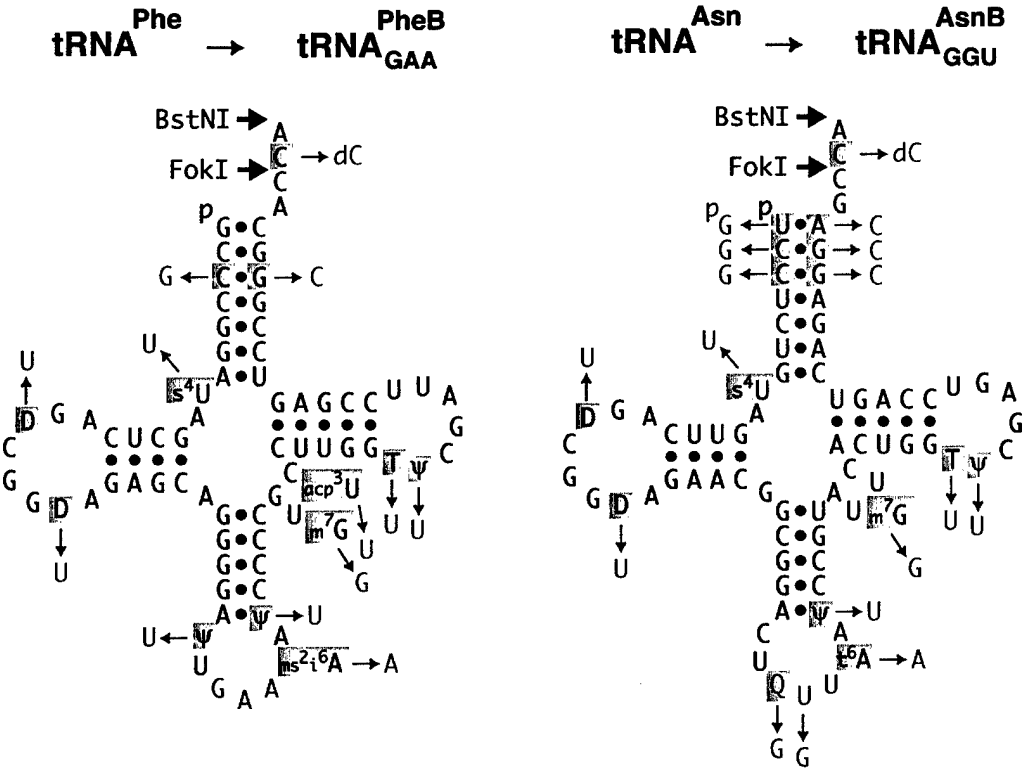



Figure 1. Left panel: our *in vitro* transcribed $tRNA^{PheB}$ adaptor. Right panel: our *in vitro* transcribed $tRNA^{AsnB}$ adaptor. Differences from the natural tRNAs are indicated in blue.

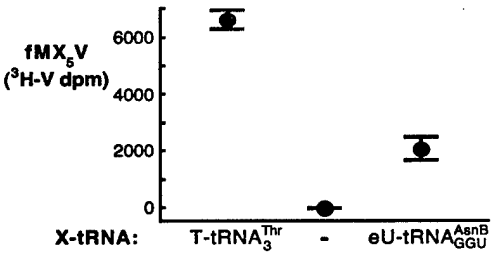
**Translating mRNA into a specific peptidomimetic polymer.** We used a chemically aminoacylated eU-tRNA$^{AsnB}_{GGU}$ adaptor (Fig. 1) to translate mRNA $MT_5V$ (Fig. 2a) to test for site-specific incorporation of several adjacent unnatural amino acids. This combination of adaptor and template allowed us to compare the efficiency of synthesis of the unnatural $fM(eU)_5V$ product to that of the natural $fMT_5V$ product, synthesized by translation of the same template with the native Thr-tRNA$_3^{Thr}$ (Fig. 2b, green). When eU-tRNA$^{AsnB}_{GGU}$ was substituted for Thr-tRNA$_3^{Thr}$ (Fig. 2b, blue), five adjacent eU's were incorporated into $fM(eU)_5V$ product at approximately 30% overall yield in comparison with $fMT_5V$, assuming similar recovery of $fMT_5V$ and $fM(eU)_5V$ during analysis. Control translations (Fig. 2b, red) without a cognate aa-tRNA for the T codon did not incorporate $^3$H-valine, confirming the specificity of decoding. HPLC analysis of the $fM(eU)_5V$ translation revealed comigration of the product with authentic $fM(eU)_5V$ marker prepared by chemical synthesis (Fig. 2c).

**a**

**mRNA $MT_5V$**

```
5' ... AUG ACC ACC ACC ACC ACC GUU GAA UUC C 3'
       fM   T   T   T   T   T   V
       fM  eU  eU  eU  eU  eU   V
```

**b**




**c**

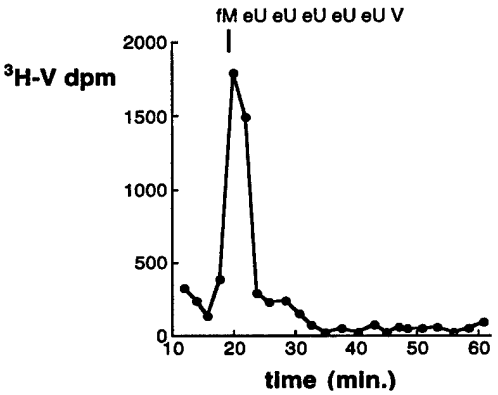



Figure 2. Translations incorporating five adjacent unnatural amino acids site-specifically. **a**, mRNA sequence, encoded natural translation product without Glu-tRNA$^{Glu}$ (green), and encoded unnatural translation product when Thr-tRNA$_3^{Thr}$ is replaced with eU-tRNA$^{AsnB}_{GGU}$ (blue). **b**, Incorporation of five adjacent eU amino acids. Positive control translations (green) contained the purified ribosomes and factors, mRNA $MT_5V$, fMet-tRNA$_i^{fMet}$, ~3 μM Thr-tRNA$_3^{Thr}$ and $^3$H-labeled-Val-tRNA$_1^{Val}$. In other translations, natural Thr-tRNA$_3^{Thr}$ was omitted (negative controls in red) or replaced with ~3 μM eU-tRNA$^{AsnB}_{GGU}$ (blue). Product values (d.p.m. after subtraction of background d.p.m. obtained in control translations lacking mRNA) represent three experiments performed on three different occasions with three different preparations of eU-tRNA$^{AsnB}_{GGU}$. Bars indicate standard deviations. X is the amino acid variable. **c**, HPLC analysis of a replicate of the translations performed with eU-tRNA$^{AsnB}_{GGU}$ in b. Radiolabeled translation reaction was treated with alkali, mixed with authentic unlabeled marker peptide ($fM(eU)_5V$ dissolved in 88% formic acid) and analyzed by reversed phase HPLC on a C-18 column. The elution position of the marker peptide is indicated above the chromatogram.

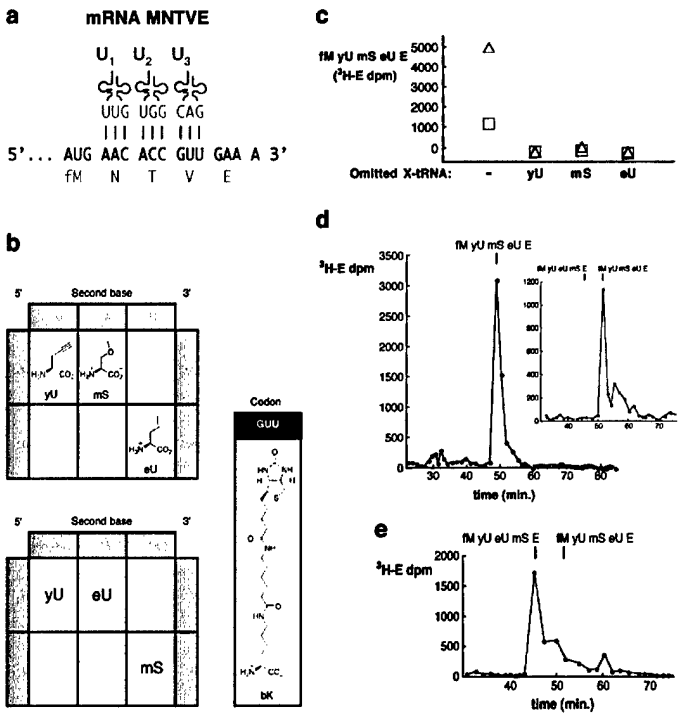



Figure 3. Translations incorporating three adjacent different unnatural amino acids by redesigning arbitrarily chosen codons. **a**, Redesign of N, T and V codons (black) of the universal genetic code to encode unnatural amino acids ($U_{1-3}$) of our choosing by mutating the anticodons of $tRNA^{PheB}_{GAA}$ and $tRNA^{AsnB}_{GGU}$ (Fig. 1). The template shown (potentially encoding the natural translation product fMNTVE, green) was synthesized to test for the adjacent incorporation of three different unnatural amino acids using the indicated synthetic adaptors. **b**, Rudimentary new genetic codes. In the blue code (top), the five-codon mRNA (a) is translated to give the product fM-yU-mS-eU-E. **c**, Dependence on each unnatural aa-tRNA for synthesis of fM-yU-mS-eU-E. (a) using either charged $tRNA^{AsnB}$ substrates (triangles) or charged $tRNA^{PheB}$ substrates (squares). **d**, HPLC analysis of a replicate of the translation performed with the unnatural AsnB aa-tRNAs (c). The elution positions of the marker peptides are indicated above the chromatograms. **e**, HPLC analysis (as in d inset) of a translation of the same mRNA using the purple genetic code (b middle) and $tRNA^{AsnB}$ adaptors, demonstrating synthesis of fM-yU-eU-mS-E.

**Creation of rudimentary genetic codes.** To test the feasibility of creating rudimentary genetic codes, we designed two sets of three adaptors based on the AsnB and PheB tRNA bodies to synthesize a template-encoded product with three adjacent different unnatural amino acids. For this purpose, we treated the anticodon as a modular unit separable from the tRNA body, substituting anticodons capable of recognizing N, T, and V codons into each tRNA body (Fig. 3a). We charged each adaptor chemically with an unnatural amino acid, and used either the AsnB set or the PheB set of chemically charged tRNAs to translate the five-codon template (Fig. 3a) according to our blue genetic code (Fig. 3b, top). The encoded fM-yU-mS-eU-E product is indeed synthesized by translation of the mRNA template with our chemically acylated $tRNA^{AsnB}$ adaptors, based on the incorporation of radioactivity into product (Fig. 3c, blue triangle), omission experiments (see below), and on the comigration on HPLC with authentic synthetic peptide (Fig. 3d). We further examined the modularity and specificity of the approach by translating the same mRNA (Fig. 3a) using the purple genetic code (Fig. 3b, bottom) to synthesize the closely related sequence fM-yU-eU-mS-E. This translation required the preparation of two new AsnB tRNA body-unnatural amino acid combinations, and yielded the expected fM-yU-eU-mS-E product, as judged by co-migration with authentic marker peptide on HPLC (Fig. 3e) and omission experiments (see below).

The lack of read-through by non-cognate tRNAs in more stringent experiments, in which each chemically aminoacylated tRNA was individually omitted, demonstrates the specificity of decoding in these translation reactions (Fig. 3c, red symbols; data for fM-yU-eU-mS-E not

shown). Using the $tRNA^{AsnB}$ adaptors, fM-yU-mS-eU-E is produced in ~55% yield (Fig. 3c, blue triangle) when compared to the amount of product translated from mRNA MVE with natural aa-tRNA substrates (Fig. 3c legend). In contrast, the relative yield was only 15% using the $tRNA^{PheB}$ adaptors (Fig. 3c, blue square), attributable to certain lower-yield individual incorporations (not shown). Lower yields are also observed with the bulky biotinyllysine derivative (Fig. 3b, right), even with a $tRNA^{AsnB}$ adaptor (20% yield, as measured by binding to avidin (Forster et al., 2001). In practice, therefore, it is advisable first to test each charged tRNA individually, as is currently done with crude systems.

These studies show that several, adjacent, arbitrarily-chosen codons can be specifically reassigned to unnatural amino acids when interfering RSs and aa-tRNAs are excluded from translations, dramatically expanding the demonstrated synthetic capabilities of the ribosome. The plasticity of translation illustrated by our studies supports the notion that the universality of the genetic code is primarily due to intrinsic constraints imposed not by the core translation apparatus but rather by RSs and the rest of the proteome. This idea is consistent with the known greater divergence of mitochondrial genetic codes, which encode very few proteins.

Coded synthesis of peptidomimetics enables new applications. For example, peptidomimetic polymers incompatible with solid phase synthesis may now be accessible. Generalization from our experiments opens up the possibility of engineering long polymers or perhaps full-length proteins with unnatural amino acids at many positions. Our synthetic scope might be extended in the future by altering the specificity of the components of the translation apparatus, such as EF-Tu, by mutation.

The design of new codes provides a novel route to create genetically-selectable, combinatorial, peptidomimetic libraries for the discovery of small-molecule ligands for targets like Muc-1, complementing other potential genetic approaches for generating degradation-resistant lead compounds, like mirror-image ligand display and non-ribosomal peptide synthesis. Theoretically, a triplet code of 64 codons, read with G-U wobble base-pairing at the third position, can be reassigned to up to 32 different amino acids, enabling the creation of extremely diverse encoded libraries of small peptidomimetics by "pure translation display." With a large enough library size, it may ultimately be possible to select drug candidates directly (compare the orally available, 11-residue cyclic peptide cyclosporin A) when building blocks like N-methyl amino acids are chosen to encode pharmacologically desirable properties such as protease-resistance and membrane permeability.

*Task 2.* Evaluate binding of the D –peptide enantiomer of the selected ligand(s) to monomeric and oligomeric forms of the native (L) enantiomer of the 20-residue epitope from the DF3 glycoprotein (months 18-30)

This task is contingent on the successful identification by phage display of a peptide ligand for the D-MUC-1 target. Because we were unable to identify a peptide ligand for the D-MUC-1 target by phage display, all of our experiemental effort has been committed to the development of technology for display of peptide or peptidomimetic libraries using our purified system for template directed peptide synthesis by ribosomes. Since our purified translation system shows promising results for use in the synthesis of oligopeptides containing unnatural amino acids, we

have devoted our full effort to the development of this system for purified ribosome display of peptides and peptidomimetics.

*Task 3.* Investigate whether the selected ligand binds specifically to cells expressing the underglycosylated form of the DF3 glycoprotein (months 24-36)

No progress thus far. No peptide ligands that bind to the MUC-1 target have yet been identified.

**KEY RESEARCH ACCOMPLISHMENTS:**

**First period (7/00-6/01)**

Demonstration that the diversity encoded in two different phage display libraries is insufficient to provide a selectable ligand for the MUC-1 target.

Reconstitution of oligopeptide synthesis from purified components.

Template-directed incorporation of a single unnatural amino acid using the reconstituted translation system.

**Previous period (7/01-6/02)**

Preparation of all 20 aminoacyl tRNA synthetases to enable charging of tRNA substrates with native cognate amino acids.

Demonstration that extended polypeptides of length up to 100 amino acids can be synthesized using the purified translation system.

Template-directed incorporation of an unnatural amino acid using the reconstituted translation system.

**Current Period (7/02-6/03)**

Template-directed synthesis of several different peptidomimetics that contain multiple adjacent unnatural amino acids.

Verification of the peptidomimetic identities by co-migration on HPLC with authentic standards.

**REPORTABLE OUTCOMES:**

Publications

Forster, A.C., Weissbach, H. and Blacklow, S.C. A Reconstitution of Oligopeptide Synthesis From Purified *E. coli* Components Using Five His-Tagged Initiation and Elongation Factors and the Epsilon Enhancer Sequence. (2001) *Analytical Biochemistry*, **297**, 60-70.

Forster A.C., Tan Z., Nalam M.N., Lin H., Qu H., Cornish V.W., and **Blacklow S.C.** Programming peptidomimetic syntheses by translating genetic codes designed *de novo*. (2003) *Proc Natl Acad Sci 100*, 6353-6357 (Appendix 1).

## CONCLUSIONS:

Two limitations inherent in phage display are the relatively small library size (less than $10^9$) and the constraint that the building blocks of the library be restricted to the 20 naturally-occurring amino acids. These constraints likely prevented us from identifying ligands for the MUC-1 target from two different phage display libraries, and we have therefore been compelled to seek an alternative method for the presentation of larger and more diverse libraries of potential ligands to the MUC-1 target. The purified translation system we have developed has the potential to overcome both restrictions encumbering the existing phage display methodology because the library size in our method can be up to $10^6$ times larger and the molecular diversity of the building blocks is not restricted to the 20 naturally-occurring amino acids.

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Forster A.C., Tan Z., Nalam M.N., Lin H., Qu H., Cornish V.W., and Blacklow S.C. Programming peptidomimetic syntheses by translating genetic codes designed *de novo*. (2003) *Proc Natl Acad Sci 100*, 6353-6357.

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**APPENDICES:**

Forster A.C., Tan Z., Nalam M.N., Lin H., Qu H., Cornish V.W., and **Blacklow S.C.** Programming peptidomimetic syntheses by translating genetic codes designed *de novo*. (2003) *Proc Natl Acad Sci 100*, 6353-6357 (Appendix 1).

# Programming peptidomimetic syntheses by translating genetic codes designed *de novo*

**Anthony C. Forster*†, Zhongping Tan‡, Madhavi N. L. Nalam*, Hening Lin‡, Hui Qu*, Virginia W. Cornish‡, and Stephen C. Blacklow*†**

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**Although the universal genetic code exhibits only minor variations in nature, Francis Crick proposed in 1955 that "the adaptor hypothesis allows one to construct, in theory, codes of bewildering variety." The existing code has been expanded to enable incorporation of a variety of unnatural amino acids at one or two nonadjacent sites within a protein by using nonsense or frameshift suppressor aminoacyl-tRNAs (aa-tRNAs) as adaptors. However, the suppressor strategy is inherently limited by compatibility with only a small subset of codons, by the ways such codons can be combined, and by variation in the efficiency of incorporation. Here, by preventing competing reactions with aa-tRNA synthetases, aa-tRNAs, and release factors during translation and by using nonsuppressor aa-tRNA substrates, we realize a potentially generalizable approach for template-encoded polymer synthesis that unmasks the substantially broader versatility of the core translation apparatus as a catalyst. We show that several adjacent, arbitrarily chosen sense codons can be completely reassigned to various unnatural amino acids according to *de novo* genetic codes by translating mRNAs into specific peptide analog polymers (peptidomimetics). Unnatural aa-tRNA substrates do not uniformly function as well as natural substrates, revealing important recognition elements for the translation apparatus. Genetic programming of peptidomimetic synthesis should facilitate mechanistic studies of translation and may ultimately enable the directed evolution of small molecules with desirable catalytic or pharmacological properties.**

The extraordinary synthetic capability of the translation apparatus, with its wide substrate diversity, capacity to synthesize long polymers, and genetic encodability using adaptors (F. Crick, quoted in ref. 1), has long made it an attractive target for biosynthetic engineering. Nevertheless, rewriting the central dogma in biology to enable information flow from nucleic acid templates to polymers of unnatural amino acids in a controllable and generalizable manner has not been realized, and decades of research have not established its feasibility. Thus, attempting this goal has value, apart from its potential applications, in testing our understanding of translation.

Early work, in which the amino acid moiety of natural aminoacyl-tRNAs (aa-tRNAs) was chemically modified after charging, showed that sense codons can be partially reassigned to either a different standard amino acid (2) or an $\alpha$-hydroxy acid (3), ultimately leading to synthesis of polymers of indeterminate length containing a random mixture of ester and amide linkages (4). Although these studies suggested the potential of harnessing translation for synthesis of encoded unnatural polymers, the approach is restricted to chemically accessible derivatives of natural aa-tRNAs and also suffers from substantial competition with amino acids in the translation extract (3).

Nonsense-suppressing aa-tRNAs (5–8), synthesized completely *in vitro* chemoenzymatically (6, 9–11) and engineered for resistance to proofreading and recharging by the aa-tRNA synthetases (RSs), have found wide utility for the specific incorporation of a large variety of unnatural amino acids at a single site per protein (12) but are restricted to use at a maximum of three stop codons. The suppression approach has been extended to rarely used sense codons by combining frameshifting aa-tRNAs with mRNAs containing extra downstream stop codons to terminate nonframeshifted products (13). With frameshifting aa-tRNAs, two nonadjacent codons have been reassigned in one mRNA (14–16), but extension to more than two codons will be restricted to the most rarely used of the 61 sense codons because of competition with natural aa-tRNAs and will require complicated overlapping reading-frame designs. In addition, extension of frameshift suppression for use at adjacent sites, necessitating the positioning of adjacent unnatural anticodons of more than three bases each on the ribosome, is likely to be problematic. Moreover, the engineering of new tRNA anticodons must circumvent inadvertent recognition by the RSs, because the anticodon is a major recognition element (17).

Efficiencies of successful single nonsense or frameshift suppressions with unnatural amino acids are frequently below 50% (12, 14–16, 18), theoretically incompatible with appreciable product synthesis if several incorporations are required, and many unnatural amino acids fail to incorporate at all (12, 18). Although the inefficiencies may be explained in part by competition with endogenous release factors (19, 20), RSs and aa-tRNAs (15), or by the use of a suboptimal suppressor tRNA (21), additional explanations are required for the dramatic differences observed between different unnatural amino acids carried by the same suppressor tRNA (12) or between different tRNA bodies carrying the same amino acid (21). Presumably, such differences alter recognition by elongation factor (EF)-Tu and/or the ribosome (see *Discussion*).

It was hypothesized that synthetic limitations with unnatural amino acids might be largely overcome by excluding the factors and activities leading to competition in translation (22). Indeed, translations performed according to these principles incorporated biotinylated lysine from a native $tRNA^{Lys}$ adaptor (22). However, in combining such a purified system (22, 23) with chemoenzymatically synthesized substrates to facilitate switching of amino acid identity and codon specificity, a concern is the potential deleterious effect of using tRNAs without native nucleoside modifications. Information on such effects is limited because chemoenzymatically synthesized substrates have been typically used in crude charging and/or translation systems known to contain tRNA modification activities (24). If unnatural substrate features are rejected by the translational machinery, efficiencies may not be improved simply by provision of longer times for incorporation. Here, we combine a purified translation system free of RSs with chemoenzymatically synthesized nonsuppressor aa-tRNA substrates to explore the versatility of translation in a potentially generalizable manner.

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Abbreviations: EF, elongation factor; IF, initiation factor; aa-tRNA, aminoacyl-tRNA; RS, aa-tRNA synthetase; $tRNA^{minusCA}$, tRNA from which the 3′-terminal CA has been deleted; $x\text{-}tRNA^{y}_{z}$, $x$ = charged amino acid, $y$ = amino acid specificity of either the natural isoacceptor or the natural isoacceptor on which the chemoenzymatic sequence is based, and $z$ = either the natural isoacceptor designation or the anticodon sequence (5′ to 3′) of chemoenzymatic tRNA sequence.

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## Materials and Methods

**Substrates.** Synthetic genes were cloned to enable *in vitro* synthesis of $tRNA^{minusCA}$ species (from *Fok*I-cut templates) for ligation to an aa-pdCpA or synthesis of full-length tRNAs (from *Bst*NI-cut templates). The tRNA sequences contained substitutions at their 5′ and 3′ termini to maintain the secondary structure of the aminoacyl stems while enabling efficient transcription initiation at the first nucleotide with GMP by T7 RNA polymerase. Nvoc-aa-pdCpA derivatives of eU (25), mS (26), bK (27), and yU[§] (Fig. 3*b*) were prepared and ligated to $tRNA^{minusCA}$ species by using general methods (11). The concentrations of Nvoc-aa-tRNA ligation products were estimated by urea polyacrylamide gel electrophoresis at pH 5 (ligation of certain aa-pdCAs required a concentration of T4 RNA ligase (New England Biolabs) severalfold higher than that recommended (11); only efficient ligations were used). Natural aa-tRNAs were prepared from pure isoacceptors (Subriden RNA, Rolling Bay, WA) as described (22) or with pure recombinant RSs (20). The specific activities of $^3H$-labeled amino acids were 21,400 (Fig. 2*b*), 14,600 (Fig. 2*c*), and 16,900 (Fig. 3) dpm/pmol.

**Translations.** mRNAs and translation mixes were prepared as described (22), except that translation components differed by omission of polyethylene glycol, addition of His-tagged EF-Ts (28), further purification of initiation factor (IF)2 by gel-filtration chromatography, and additional washing of ribosomes. Ribosome salt washes were as described (22), except that an additional high-speed spin of 1 min preceded the final pelleting of the four-times-washed ribosomes to remove residual insoluble material. Ribosomes and factors were not contaminated with RSs or proteases, as measured by charging of total tRNA (Sigma) with 15 $^{14}C$-labeled amino acids (New England Nuclear) and by stability of peptides. Macromolecular concentrations in translations were adjusted slightly to give 0.5 $\mu$M each of IF1, IF2, IF3, EF-G, and EF-Ts, 2.5 $\mu$M EF-Tu, four-times-washed ribosomes at 0.029 $A_{260}$ unit/$\mu$l [27 nM estimated to be active (22)], 1 $\mu$M mRNA, 0.2 $\mu$M fMet-$tRNA_i^{fMet}$, and 0.5 $\mu$M (unless otherwise indicated) each elongator aa-tRNA, and translations were typically performed at 37°C for 30 min without preincubation. Translations analyzed by cation-exchange (treatment with alkali, acidification, then minichromatography to separate anionic formylated peptides from unformylated amino acids) were all performed on a 1-pmol scale with respect to limiting input fMet-$tRNA_i^{fMet}$, whereas the scales varied for translations analyzed by HPLC. Peptide markers were synthesized on an Advanced Chemtech peptide synthesizer from commercial reagents.

## Results

**A Purified RS-Free Translation System with Modular tRNA Adaptors.** Our system (Fig. 1*a*) was constructed from ribosomes purified exhaustively to remove measurable contaminating RS charging activities (see *Materials and Methods*), recombinant translation factors (22), *in vitro*-synthesized mRNAs, *in vitro*-charged native tRNA isoacceptors, and chemoenzymatically synthesized aa-tRNAs. In pilot studies, a $tRNA^{Asn}$-based chemoenzymatically synthesized elongator tRNA that was arbitrarily chosen (termed $tRNA^{AsnB}_{GGU}$, where the subscript refers to the anticodon; Fig. 1*b*) was nonenzymatically charged with eU (structure shown in Fig. 3*b*) and assayed in the purified translation system for single incorporation directed by mRNA MTV (see *Materials and Methods*). It was comparable in specificity and efficiency to the natural Thr-$tRNA_3^{Thr}$, even at 0.5 $\mu$M (a concentration 20 to 40-fold lower than typically needed in crude translation systems; refs.11 and 31), alleviating concerns that altering the tRNA body (Fig. 1*b*) or charging with the unnatural amino acid eU might be problematic. Control translations with unacylated full-length $tRNA^{AsnB}_{GGU}$ in place of eU-$tRNA^{AsnB}_{GGU}$ did not synthesize any

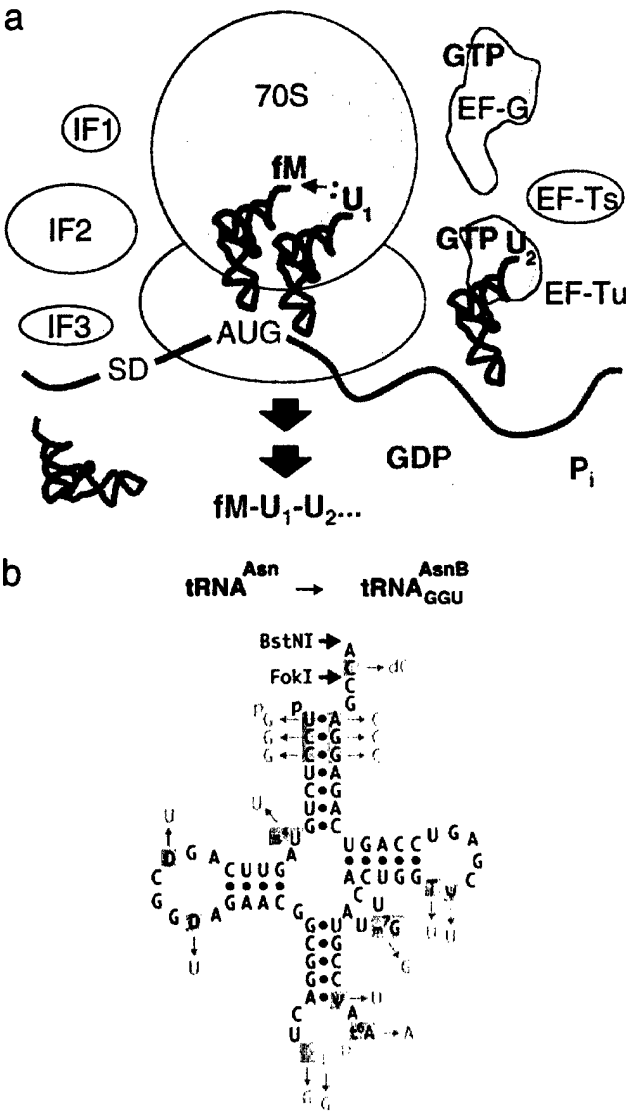


**Fig. 1.** Our purified substrate-based translation system lacking RS activities. (*a*) The core translation machinery (blue) is depicted incorporating multiple unnatural amino acids ($U_1$, $U_2$, . . . ) into peptidomimetic product. *Escherichia coli* served as the source for our natural components. IF1, IF2, IF3, His-tagged initiation factors; EF-Tu, EF-Ts, EF-G, His-tagged elongation factors. An mRNA template containing a Shine and Dalgarno ribosome binding site (SD) is colored purple, substrates are green, and products are red. Regeneration of GTP from GDP is catalyzed by pyruvate kinase using phospho*enol*pyruvate substrate (data not shown). After translation, peptide products are released from the peptidyl-tRNAs by base-catalyzed hydrolysis [termination factors were omitted from the system for simplicity and because rapid product release would be undesirable for ribosome display experiments (29)]. (*b*) Natural *E. coli* $tRNA^{Asn}$ (ref. 30; black; the anticodon is purple) and our synthetic ligated derivative, $tRNA^{AsnB}$ (differences from the natural tRNA in blue).

[§]In our nomenclature for individual nonstandard amino acids, amino acids other than the standard 20 are represented by an uppercase letter and a lowercase letter prefix. The uppercase letter refers to the standard abbreviation of the related natural amino acid side chain and the prefix refers to the nonstandard functional group. When an unnatural amino acid side chain is unrelated to a natural one, we designate the uppercase letter part as U for unrelated unnatural. Thus, *formyl*methionine is fM, *biotinyl*lysine is bK, *O-methyl*serine is mS, and 2-amino-4-pente*noic* acid and 2-amino-4-penty*noic* acid are eU and yU, respectively (eU is also known as allylglycine; structures are shown in Fig. 3*b*). Nvoc indicates the amino-protecting group 6-nitroveratryloxycarbonyl.

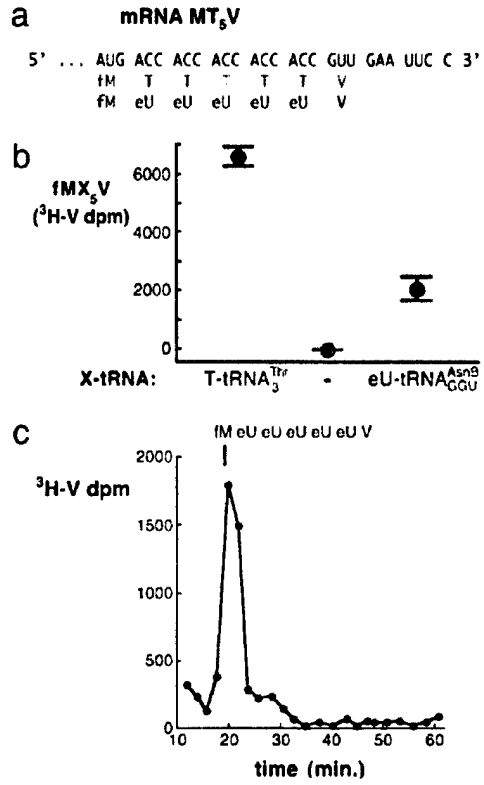


**Fig. 2.** Translations incorporating five adjacent unnatural amino acids site-specifically. (*a*) mRNA sequence, encoded natural translation product without Glu-tRNA$^{Glu}$ (green), and encoded unnatural translation product when Thr-tRNA$_3^{Thr}$ is replaced with eU-tRNA$^{AsnB}_{GGU}$ (blue). (*b*) Incorporation of five adjacent eU amino acids. Positive control translations (green) contained the purified ribosomes and factors, mRNA $MT_5V$, fMet-tRNA$_i^{fMet}$, ≈3 μM Thr-tRNA$_3^{Thr}$, and $^3$H-labeled-Val-tRNA$_1^{Val}$. In other translations, natural Thr-tRNA$_3^{Thr}$ was omitted (negative controls in red) or replaced with ≈3 μM eU-tRNA$^{AsnB}_{GGU}$ (blue). Product values (dpm after subtraction of background dpm obtained in control translations lacking mRNA) represent three experiments performed on three different occasions with three different preparations of eU-tRNA$^{AsnB}_{GGU}$. Bars indicate standard deviations. X, amino acid variable. (*c*) HPLC analysis of a replicate of the translations performed with eU-tRNA$^{AsnB}_{GGU}$ in *b*. Radiolabeled translation reaction was treated with alkali, mixed with authentic unlabeled marker peptide [fM(eU)$_5$V dissolved in 88% formic acid], and analyzed by reversed-phase HPLC on a $C_{18}$ column. The chromatogram shows a 27–71% acetonitrile/water linear gradient in the presence of 0.1% trifluoroacetic acid. The elution position of the marker peptide is indicated above the chromatogram. Peptide products were not detectable on a 2–32% acetonitrile/water gradient used for resolving less hydrophobic peptides such as $fMT_5V$ (ref. 22; data not shown).

full-length peptide, confirming that eU was indeed incorporated into products (data not shown).

**Translating mRNA into a Specific Peptidomimetic Polymer.** We then used the chemically aminoacylated eU-tRNA$^{AsnB}_{GGU}$ adaptor (Fig. 1*b*) to translate mRNA $MT_5V$ (Fig. 2*a*) to test for site-specific incorporation of several adjacent unnatural amino acids. This combination of adaptor and template allowed us to compare the efficiency of synthesis of the unnatural fM(eU)$_5$V product to that of the natural $fMT_5V$ product, synthesized by translation of the same template with the native Thr-tRNA$_3^{Thr}$ (Fig. 2*b*, green). When eU-tRNA$^{AsnB}_{GGU}$ was substituted for Thr-tRNA$_3^{Thr}$ (Fig. 2*b*, blue), five adjacent eUs were incorporated into fM(eU)$_5$V product at ≈30% overall yield in comparison with $fMT_5V$, assuming similar recovery of $fMT_5V$ and fM(eU)$_5$V during analysis. Control translations (Fig. 2*b*, red) without a cognate aa-tRNA for the T codon did not incorporate [$^3$H]valine, confirming the specificity of decoding. HPLC analysis of the fM(eU)$_5$V translation revealed comigration of the product with authentic fM(eU)$_5$V marker prepared by chemical synthesis (Fig. 2*c*).

**Creation of Unnatural Genetic Codes.** To test the feasibility of creating rudimentary genetic codes, we constructed an mRNA containing three adjacent different test codons (AAC, ACC, and GUU) and also constructed the necessary two additional tRNA adaptors by using the tRNA$^{AsnB}$ body (Fig. 3*a*). Importantly, the test codons had been chosen arbitrarily and are present in three different codon boxes (standard groupings) of the universal genetic code, whereas the adaptors for their translation had been created rationally, differing only by anticodon mutations guided by the rules of codon–anticodon base pairing. We charged each adaptor chemically with an unnatural amino acid according to our blue genetic code (Fig. 3*b Upper*) and used these substrates for translation of the five-codon template (Fig. 3*a*). The encoded fM-yU-mS-eU-E product is indeed synthesized, based on the incorporation of radioactivity into product (Fig. 3*c*, blue open triangle), omission experiments (see below), and the comigration on HPLC with authentic synthetic peptide (Fig. 3*d*). We further examined the modularity and specificity of the approach by translating the same mRNA (Fig. 3*a*) by using the purple genetic code (Fig. 3*b Lower*) to synthesize the closely related sequence fM-yU-eU-mS-E. This translation required the preparation of two new combinations of unnatural amino acids and tRNA$^{AsnB}$ bodies and yielded the expected fM-yU-eU-mS-E product, as judged by incorporation of radioactivity into product (Fig. 3*c*, purple filled triangle) and comigration with authentic marker peptide on HPLC (Fig. 3*e*). The lack of read-through by noncognate aa-tRNAs in more stringent experiments, in which each chemically aminoacylated tRNA was individually omitted, further demonstrates the specificity of decoding in these translation reactions (Fig. 3*c*, red triangles).

The yield of fM-yU-mS-eU-E is ≈55% when compared with the amount of product translated from mRNA MVE with natural aa-tRNA substrates (Fig. 3*c* legend), and the amount of fM-yU-eU-mS-E is lower (Fig. 3*c*). When the tRNA$^{AsnB}_{GGU}$ adaptor is chemically aminoacylated with the bulky biotinyllysine derivative (Fig. 3*b Right*) and tested for a single incorporation, the yield is only 20% [measured by binding to avidin (22); data not shown]. In practice, therefore, it is advisable first to test each charged tRNA individually, as is currently done with crude systems. It is also apparent that even our tRNAs charged with smaller unnatural amino acids give somewhat lower yields of products in translations requiring three to five adjacent unnatural amino acid incorporations in comparison with control translations containing only natural aa-tRNAs.

## Discussion

Prior engineering of translation has been limited to the specific reassignment of the amino acid identity of only a small subset of the 64 codons at two nonadjacent codon positions within an mRNA because of the inherent restrictions of suppressor tRNAs and crude translation systems. Here, our combination of a purified translation system free of RSs with chemoenzymatically synthesized nonsuppressor aa-tRNA substrates enabled several, adjacent, arbitrarily chosen codons to be completely reassigned to unnatural amino acids in a potentially generalizable manner. The plasticity of translation illustrated by our studies supports the notion that the universality of the genetic code is primarily due to intrinsic constraints imposed not by the core translation apparatus but rather by RSs and the rest of the proteome. This idea is consistent with the known greater divergence of mitochondrial genetic codes, which encode very few proteins (32).

The study of translation using custom-designed substrates with our purified system (a purified "polypeptide polymerase") has advantages over crude systems. Although inefficiencies with

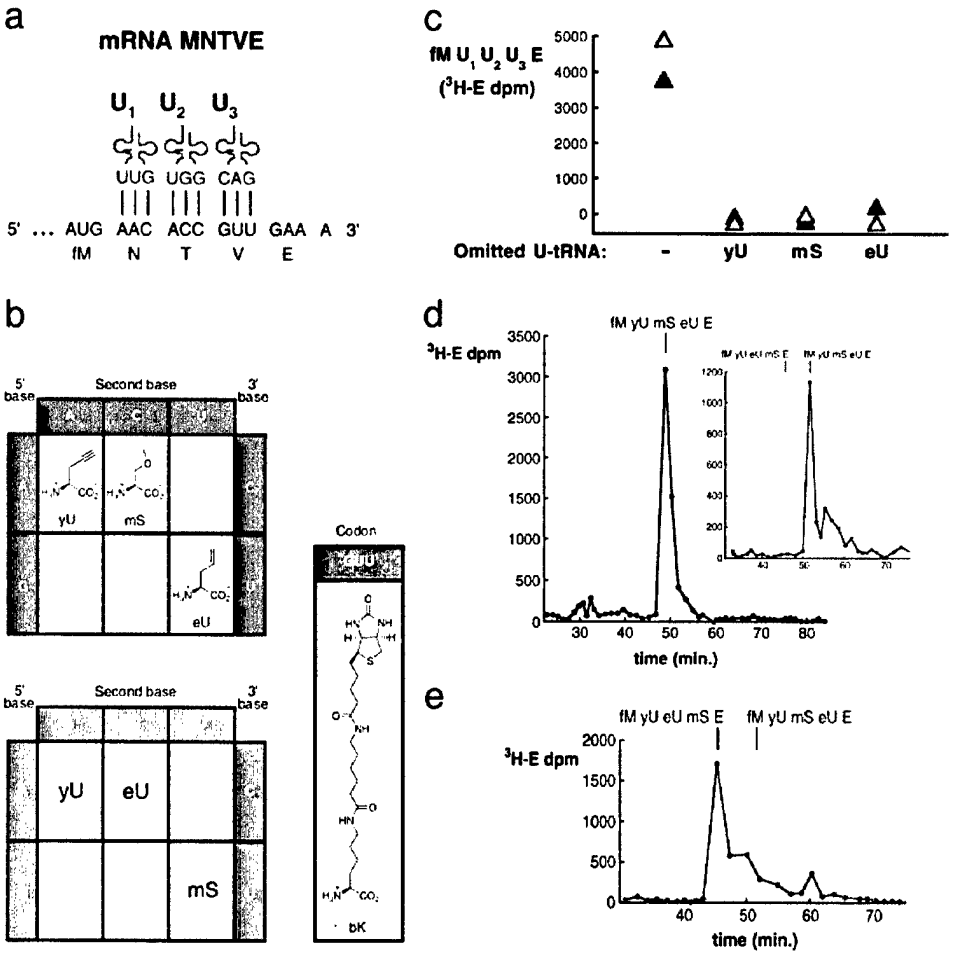


**Fig. 3.** Translations incorporating three adjacent different unnatural amino acids by reassigning arbitrarily chosen codons. (*a*) Reassignment of $N_{AAC}$, $T_{ACC}$, and $V_{GUU}$ codons of the universal genetic code to encode unnatural amino acids ($U_{1-3}$) of our choosing. Two additional adaptors, termed $tRNA^{AsnB}_{GUU}$ and $tRNA^{AsnB}_{GAC}$, were constructed to give the group of three chemoenzymatically synthesized tRNAs shown, differing only in their anticodon sequences (blue). The template (black), potentially encoding the natural translation product fMNTVE (green), was synthesized to test for the adjacent incorporation of three different unnatural amino acids by using these synthetic adaptors. (*b*) Rudimentary new genetic codes. Translation of the five-codon mRNA illustrated in *a* according to the blue code (*Upper*) would give the product fM-yU-mS-eU-E, whereas the purple code (*Lower*) would give fM-yU-eU-mS-E. (*c*) Dependence on each unnatural aa-tRNA for synthesis of fM-yU-mS-eU-E and fM-yU-eU-mS-E. All translations contained purified ribosomes and factors, fMet-$tRNA_i^{fMet}$, and $^3$H-labeled-Glu-$tRNA_1^{Glu}$. The positive control translation (data not shown) was supplemented with mRNA MVE (22) and unlabeled Val-$tRNA_1^{Val}$, and yielded 8,600 dpm of product. The fM-yU-mS-eU-E translation (blue open triangle) was supplemented instead with mRNA MNTVE (*a*) and substrates yU-$tRNA^{AsnB}_{GUU}$, mS-$tRNA^{AsnB}_{GGU}$, and eU-$tRNA^{AsnB}_{GAC}$ (charged according to the blue genetic code), each at 1 $\mu$M, whereas control translations (red open triangles) omitted the individual unnatural aa-tRNAs listed below the *x* axis. The fM-yU-eU-mS-E translation (purple filled triangle) was also supplemented with mRNA MNTVE and yU-$tRNA^{AsnB}_{GUU}$ but differed in containing eU-$tRNA^{AsnB}_{GGU}$ and mS-$tRNA^{AsnB}_{GAC}$ (see purple genetic code), whereas control translations (red filled triangles) omitted the aa-tRNAs listed. Background dpm obtained in a translation without mRNA was subtracted. (*d*) HPLC analysis of a replicate of the complete translation performed in *c* by using the blue code. Radiolabeled translation reaction was treated with alkali, mixed with authentic unlabeled marker peptide (fM-yU-mS-eU-E), and analyzed by reversed-phase HPLC on a $C_{18}$ column using a 4–32% acetonitrile/water gradient (large plot), or mixed with two closely related markers of identical amino acid composition and analyzed by a shallower 9–14% gradient to maximize resolution (*Inset*). The elution positions of the marker peptides are indicated above the chromatograms. (*e*) HPLC analysis (as in *d Inset*) of a replicate of the complete translation performed in *c* by using the purple code, demonstrating synthesis of fM-yU-eU-mS-E.

unnatural aa-tRNAs in crude systems are at least partly due to competing activities, this cannot be the case in our system because competitors have been deliberately excluded. Thus, the interesting observation here that unnatural substrates are still less efficient than natural ones directly reveals substrate determinants necessary for efficient translation (Figs. 1*b* and 3*a*) and suggests the existence of other competing reactions [one possibility is peptidyl-tRNA drop-off from the ribosome (23, 33)]. Nevertheless, the findings that our incorporations were more efficient than generally observed in crude systems, despite using lower concentrations of unnatural aa-tRNAs and the omission of all termination factors (33), and that adjacent unnatural amino acids can be incorporated broadens the range of experimental possibilities. For example, appropriately chosen pairs of adjacent unnatural amino acids might be used to probe the chemical mechanism of ribosomal peptide bond formation.

What recognition elements have been altered by the introduction of unnatural features into our substrates? Given that the affinities of EF-Tu for native aa-tRNAs are similar, whereas its affinities for mismatched combinations of amino acids and tRNA bodies are very different, one hypothesis is that efficient delivery by EF-Tu to the ribosome of each amino acid may require matching with a tRNA body of appropriate compensatory affinity for EF-Tu (34). Alternatively, tRNA nucleoside modifications can be important for efficient translation *in vivo* (35), whereas interpretation of *in vitro* studies with unmodified

tRNAs by using crude charging or translation systems is complicated by the presence of endogenous modification activities (24). The loss of anticodon loop nucleoside modifications in many tRNAs is hypothesized to decrease anticodon–codon stability on the ribosome (36), which could lead to increased dissociation of cognate aa-tRNAs from the ribosome at both the initial selection and proofreading steps (37). Perhaps unnatural amino acid incorporation from our substrates could be improved by altering EF-Tu or the ribosome.

Our system should facilitate unambiguous definition of substrate elements that affect translational activity, including the enigmatic nucleoside modifications. Synthetic aa-tRNAs could be constructed to more closely resemble readily available natural aa-tRNAs for comparative studies, e.g., by using tRNA sequences other than that of $tRNA^{Asn}$ and unmutated tRNA sequences created by cleavage of *in vitro* synthesized precursors (38, 39). These findings may also be helpful in extending the synthetic scope of our initial system and existing suppressor systems.

Finally, we propose a potential application of our system for ligand discovery. Although the scalability of our system is not designed to provide an alternative to solid-phase peptide synthesis for preparation of individual peptides or to suppressor technology for preparation of proteins containing one or two nonadjacent unnatural amino acids, it is designed to produce the largest screenable libraries of small peptidomimetics. Theoretically, translation with 20 different aa-tRNAs of mRNA templates containing 10 random codons gives a library of $10^{13}$ different peptidomimetics (we synthesized $10^{12}$ peptidomimetics in a 70-$\mu$l translation, a fraction of which was analyzed in the experiment depicted in Fig. 2*c*) when $\approx$0.01 nmol of each aa-tRNA is incorporated (the typical research scale for aa-tRNA preparation is 1 nmol). Recycling of aa-tRNAs is also plausible by extrapolation of generalizable methods for engineering new synthetase specificities (8, 40, 41). Our approach therefore provides another route to create libraries for the discovery of small-molecule ligands or stereospecific catalysts (42), complementing other potential approaches for generating genetically encoded degradation-resistant lead compounds, such as mirror-image ligand display (43, 44) and nonribosomal peptide synthesis (45). The potential attraction of such approaches is that directed Darwinian evolution *in vitro* (29) is much faster than the many person-years of random chemical syntheses typically needed in industry for lead optimization, and their unrivalled library sizes may produce ligands with higher affinities. It is even conceivable that such selections could yield drug candidates directly (compare the orally available, 11-residue cyclic peptide cyclosporin A) when building blocks such as *N*-methyl amino acids are chosen to encode pharmacologically desirable properties such as protease resistance and membrane permeability.

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