Chapter 3

Incorporation of Unnatural Amino Acids into mRNA Display Libraries Using Sense Suppression

This chapter is adapted from the following reference:

Frankel, A., Millward, S.W., and Roberts, R.W. (2003) Encodamers: Unnatural Peptide Oligomers Encoded in RNA. *Chem. Biol. 10,* 1043-1050.

Abstract

Conventional display libraries are generally limited to the 20 naturally occurring amino acids. Here, we demonstrate that novel unnatural amide-linked oligomers can be constructed and encoded in an attached RNA for the purpose of mRNA display library design. To do this, we translated templates of various lengths in a rabbit reticulocyte lysate translation system modified to promote sense codon suppression. Unnatural residues were escorted to the ribosome as chemically-acylated tRNAs added to the translation mixture. Our experiments reveal that unnatural peptide oligomers ("encodamers") consisting of *N*-substituted amino acids are readily generated as mRNA-peptide fusions with excellent stepwise efficiency. The *N*-substituted polyamides have strikingly improved proteolytic stability relative to their naturally encoded counterparts. Overall, our work indicates that the ribosome can be used as a synthesis platform to generate encoded combinatorial chemistry lying far outside the universal genetic code.

Introduction

The development of therapeutic peptide ligands is an important application of combinatorial libraries. Currently, peptide ligand discovery has been explored in two prominently different modes. In biological display libraries, such as the phage display system, libraries are constructed from the 20 natural amino acids and displayed in a format where they are topologically associated with their encoding genetic material (*1*). These naturally constructed display libraries allow billions to trillions of compounds to be explored (*2*), but the disadvantage is that the encoded peptides or proteins are typically substrates for proteolysis. By contrast, chemically constructed one-bead-one-compound libraries (*3*) can utilize any unnatural amino acid monomer that can be coupled with reasonable efficiency. This chemical approach can confer improved properties such as protease resistance, but typically limits library size to 10^5 unique compounds and requires deconvolution to identify the products of selection, a process in which the technical difficulty increases with the desired complexity of the library (*4*).

The advent of totally *in vitro* display libraries, including ribosome display (*5*, *6*), tRNA display (*7*), and mRNA display (*8*), open the possibility of creating unnatural libraries encoded in RNA because sense codons can be suppressed with arbitrary amino acids without concern for host viability (*9*). Additionally, these *in vitro* display approaches allow even a single functional molecule to be isolated from vast molecular libraries since the encoding information can be amplified by PCR after each selection cycle. Unnatural amino acids have been inserted and selected at single sites in mRNA display libraries by nonsense suppression (*10*), sense suppression (*11*), and chemical derivatization (*12*). Creating display libraries that contain multiple consecutive insertions

of unnatural amino acids represents an important goal in unifying the benefits of natural and chemically synthesized libraries. The ability to generate polypeptides containing two or more unnatural insertions in response to either 4 base codons (*13*) or in a reconstituted translation extract (*14*) represent important steps in that direction.

The ability to create trillion-member unnatural peptide libraries with modest chain length (2 to 12 residues) would facilitate construction and selection of molecules similar to therapeutically important natural products produced by non-ribosomal peptide synthetases (NRPSs) (*15, 16*). The natural products that NRPSs generate exhibit a broad range of biological activities, likely resulting from the structural diversity and chemical complexity that they contain. These compounds are typically assembled *in vivo* by a single multidomain protein in which each domain performs one step in a multistep synthesis. Non-ribosomal peptides (NRPs) can exhibit good levels of oral bioavailability despite violating common rules of thumb (*17*). We note that the well-known "rule of five" is believed to be a poor predictor of oral bioavailability in the NRP class (*18*). The best known example is the NRP cyclosporin A (trade name "Sandimmune"; MW = 1203.6 g × mol^{-1}), a cyclic undecamer that serves as a clinically important immunosuppressant in organ transplantation and is 25-50% orally available (*19*). Cyclosporin A acts intracellularly by forming a ternary complex with cyclophilin and calcineurin (*20*). Seven of the amide linkages in cyclosporin A contain *N*-methyl substituents that likely contribute to its proteolytic resistance (*21, 22*).

Here, we have worked to create unnatural, *N*-methylated oligomers encoded in a covalently attached RNA. To do this, we have translated mRNA display templates containing 2, 5, or 10 consecutive sense codons that can be suppressed by an orthogonal,

chemically-acylated tRNA. These templates bear a 3' puromycin moiety that forms an amide linkage with the nascent peptide on the ribosome (*2, 8*). Biochemical analysis was used to examine the efficiency of synthesis, product distribution, and stability of the resulting molecules. The encoded unnatural peptide oligomers or "encodamers" generated in this study represent a new approach to encoded combinatorial chemistry with a genetic code of our choosing (Table 3.1).

Results and Discussion

We began by generating polymers of unnatural amino acids encoded as mRNA-peptide fusions. This required a subversion of the genetic code to include unnatural amino acids via codon reassignment. Previously, we demonstrated efficient sense codon-mediated incorporation of biocytin in a mRNA-peptide fusion at GUA valine codons by using chemically-acylated tRNAs (*11*). In that work, *in vitro* suppression of sense codons was performed in rabbit reticulocyte lysate partially depleted of endogenous tRNAs via column chromatography (*23*). This modified lysate allows efficient incorporation of unnatural amino acids at arbitrary codons and synthesis of corresponding mRNA-peptide fusions containing a single unnatural residue. We therefore employed this lysate as the translation platform to examine the creation of mRNA-peptide fusions containing multiple unnatural amino acid substitutions.

For this study, we designed three RNA templates containing two, five, or ten consecutive GUA codons attached to a flexible DNA linker ending with a 3'-puromycin (Figure 3.1). The templates were translated in a tRNA-depleted extract in the presence or absence of N-methyl-Phe $tRNA^{UAC}$ (NMF-$tRNA^{UAC}$), as shown in Figure 3.1, with the intention of producing Met-(*N*-methyl-Phe)$_n$ polymers covalently attached through

CODON	AMINO ACID	tRNA	ORTHOGONAL AMINO ACID
AUG	Met	$tRNA_i^{Met}$ [46]	---
GUA	Val	$tRNA^{UAC}$	Phe, *N*-methyl-Phe
GCU	Ala	$tRNA^{AGC}$	Val, Phe, *N*-methyl-Phe

Table 3.1: Reassignment of codons in this study

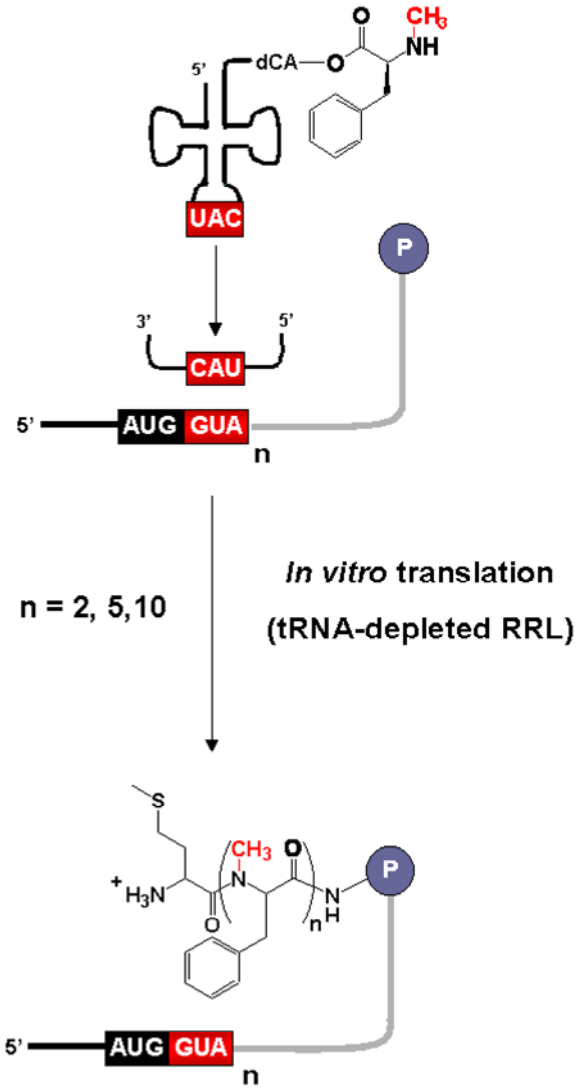

Figure 3.1: Suppression scheme at GUA codons. RNA templates containing GUA repeats (*black*) tethered by DNA (*grey*) to a 3'-puromycin (P) translated in the presence of *N*-methyl-phenylalanine-tRNA^{UAC} in tRNA-depleted rabbit reticulocyte lysate result in the formation of an *N*-methyl-Phe polymer covalently attached to its orthogonally encoded message.

puromycin to their mRNA encoded messages. The resulting products were purified and treated with RNase A so that gel mobility analysis could be used to examine the length and homogeneity of the fusions.

Figures 3.2A and 3.2B indicate that translation products are formed in both the presence and absence of exogenous NMF-tRNA^{UAC}. When fusion products are analyzed by denaturing urea-PAGE, mobility differences should reflect any variation in the translated peptide since all templates contain the same RNase-resistant F30P linker (*8*). This analysis reveals that products from the 2G and 5G templates are relatively uniform when NMF-tRNA^{UAC} is added to the translation reaction and that they run with correspondingly lower mobility than the 41P control. This observation is consistent with the formation of two- and five- residue oligopeptides on these templates, respectively. The 10G template forms a product that appears to aggregate, running as a diffuse smear under these conditions (lane 7). The product is distinct from the reaction lacking N-methyl-Phe tRNA^{UAC} (lane 6) and consistent with aggregation in the longer hydrophobic N-methyl-Phenylalanine oligomer.

Analysis of the same NMF-tRNA^{UAC} reactions in a peptide-resolving SDS tricine gel (where mobility depends both on the charged F30P portion and SDS bound to the peptide or linker; Figure 3.2B) reveals that distinct and relatively homogeneous products are formed on all of the templates tested. All three migrate with lower mobilities than the control 41P product, consistent with synthesis of two-, five-, and ten-residue N-methyl-Phe oligomers. When NMF-tRNA^{UAC} is omitted, a higher mobility series of bands are observed, consistent with the synthesis of truncated or valine-containing oligomers originating from residual endogenous Val-tRNA^{UAC}.

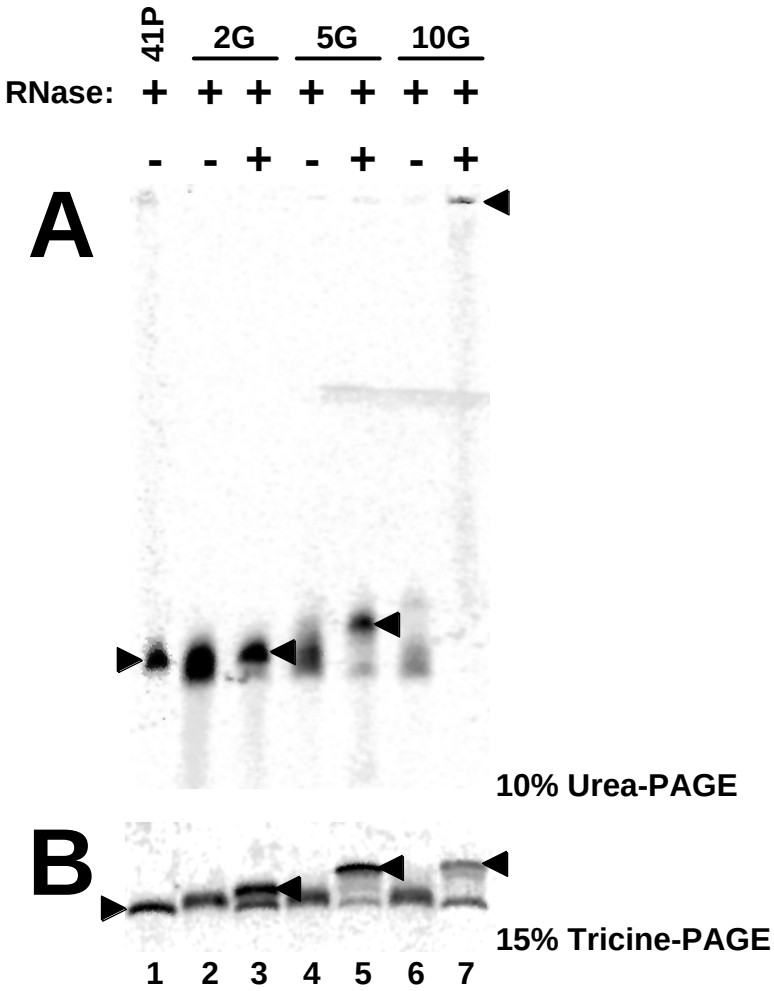

Figure 3.2: tRNA-dependence for full-length encodamer formation. Templates were translated in the presence or absence of 2 µg *N*-methyl-Phe-tRNA^{UAC}. Purified ^{35}S-labeled mRNA-peptide fusions (10 µL) were treated with 1 µg RNase A (DNase-free; Qiagen) at ambient temperature and subsequently run on either 10% Urea-PAGE (A) or 15% Tricine-PAGE (*47*) (B) for phosphorimaging. The arrows indicate full-length encodamers after RNA hydrolysis.

In an effort to show that poly(GUA) can indeed code for *N*-methyl-Phe encodamers, we subjected purified fusions to a battery of hydrolytic enzymes whose activities would facilitate the loss of the ^{35}S-Met label in the event of natural amino acid insertion while preserving the radiolabel in the unnatural oligomer. Whereas the natural fusion products are subject to proteolysis, the inclusion of NMF-tRNA^{UAC} in the reactions results in proteolytically resistant fusions (Figure 3.3). Treatment of these encodamers with chymotrypsin (apparently contaminated with RNase activity; Figure 3.3, lanes 4-12) generates the unnatural oligomer attached to its DNA linker, while treatment with proteinase K (Figure 3.3, lanes 13-18) and pepsin (data not shown) leave the full-length fusion intact. These data support the notion that the formation of proteolytically stable encodamers is dependent on the addition of exogenous NMF-tRNA^{UAC}. It should be noted that the total amount of radiolabeled fusion material decreases in all samples treated with a proteolytic enzyme (Figures 3.3A-C, lanes 7-18) relative to samples left untreated (lanes 1-6). This may be the result of 1) hydrolysis of the labile L-puromycin amide bond, and/or 2) hydrolysis of a population of fusion products containing one or more valine residues.

The observed slight heterogeneity of products in the longer encodamers (Figures 3.3B and 3.3C, lanes 3 and 6) may be reflective of the quality of the RNA template or a result of random valine insertion. To address the latter possibility we titrated the amount of NMF-tRNA^{UAC} used in the translation of the 5G template. As shown in Figure 3.4A, increasing amounts of exogenous tRNA present in the translation reactions result in encodamers with higher resistance to proteinase K digestion. This effect appears to plateau at 2 µg NMF-tRNA^{UAC}, and demonstrates that at higher concentrations of

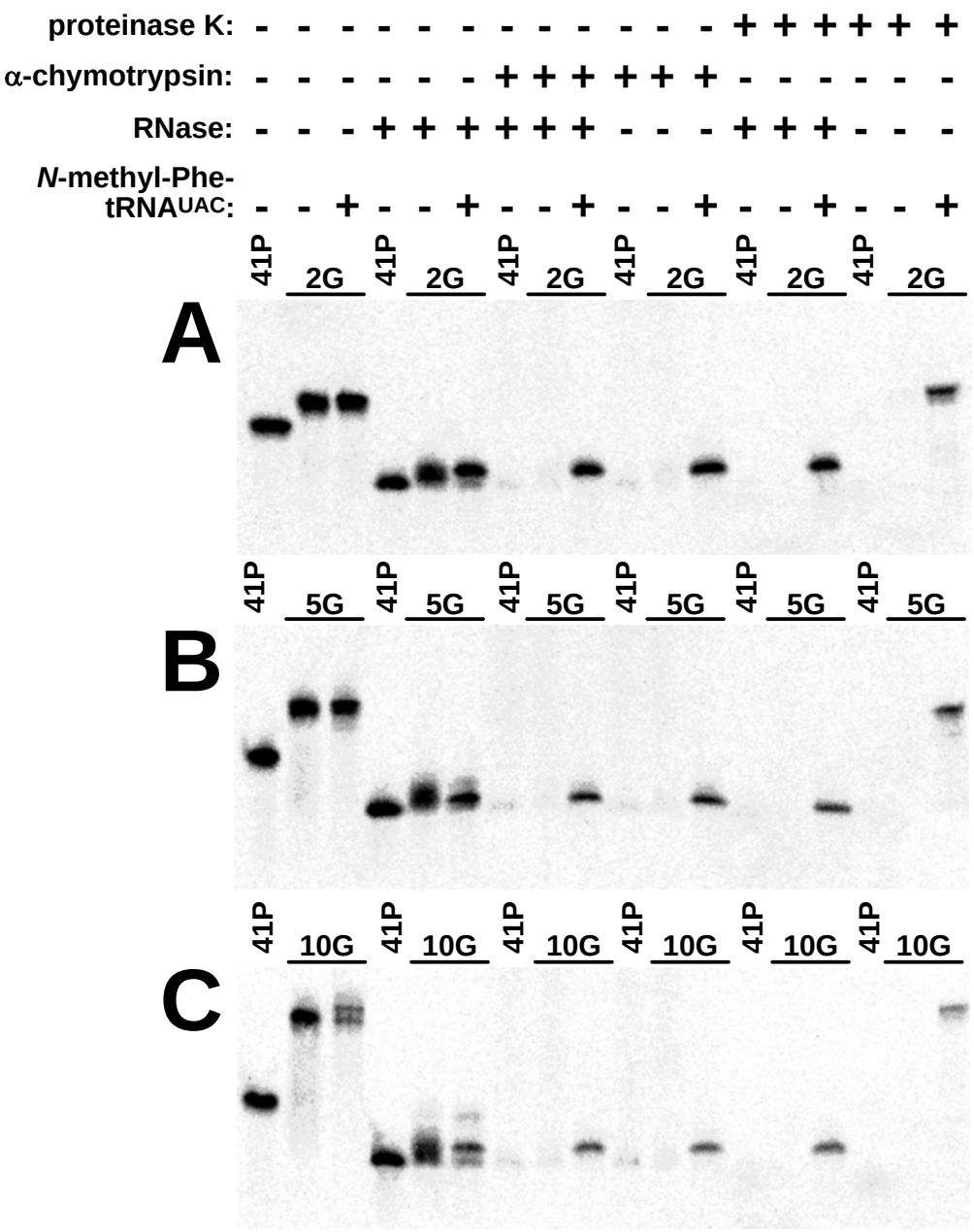

Figure 3.3: Encodamer proteolytic resistance. Templates containing GUA repeats were translated in the presence or absence of 2 µg NMF-tRNA^{UAC}. Purified ^{35}S-labeled mRNA-peptide fusions (10 µL) were treated with either 1 µg RNase A at ambient temperature, 10 µg α-chymotrypsin for 90 min at 25 ˚C with or without RNase A, or 10 µg proteinase K for 30 min at 37 ˚C with or without RNase. Panels (A) through (C) are phosphorimages of 10% Tricine-PAGE gels for templates 2G, 5G, and 10G, respectively.

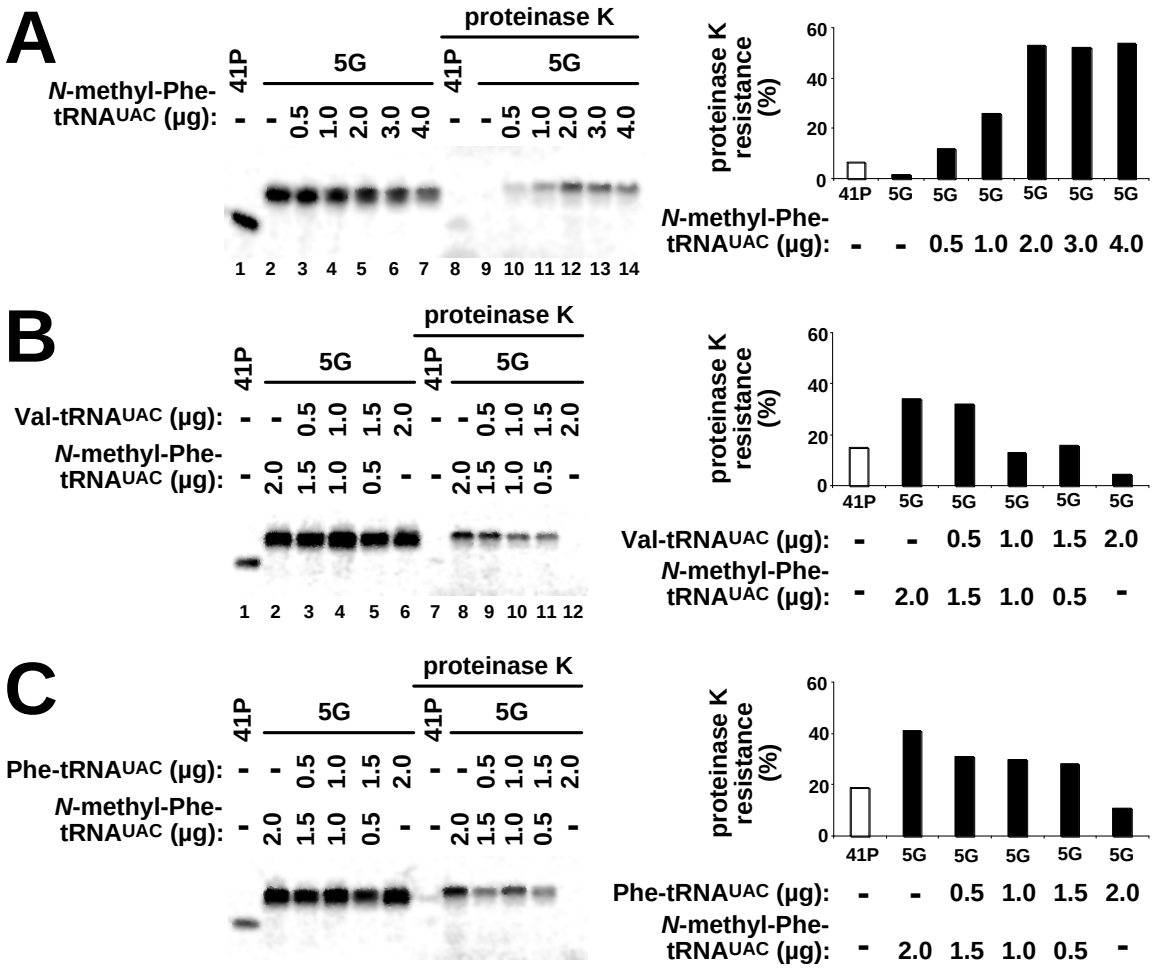

Figure 3.4: Encodamer stability is dependent upon *N*-methyl-Phe incorporation. (A) The 5G template was translated in the presence of indicated amounts of *N*-methyl-Phe-tRNA^{UAC}. Purified ^{35}S-labeled mRNA-peptide fusions (10 µL) were treated with or without 10 µg proteinase K for 60 min at 37 ˚C as indicated and subsequently run on a 10% Tricine-PAGE gel for phosphorimaging. The ratios of stable encodamers after proteinase K treatment (lanes 8-14) to the untreated encodamers (lanes 1-7) were determined by phosphorimaging and are shown in the graph (right). (B) and (C) The 5G template was translated in the presence of indicated amounts of *N*-methyl-Phe-tRNA^{UAC} and either Val-tRNA^{UAC} (B) or Phe-tRNA^{UAC} (C). The samples were treated as described in (A). The ratios of stable encodamers after proteinase K treatment (lanes 7-12) to the untreated encodamers (lanes 1-6) were determined by phosphorimaging and are shown in the graphs (right).

exogenous tRNA, insertion of the unnatural amino acid out-competes natural amino acids for the GUA codon with an approximately 30-fold enrichment for proteolysis resistance.

Although it is difficult to comment on the precise level of unnatural amino acid incorporation at a single GUA codon within the context of the 5G template, we can consider the encodamer a product of five independent suppression steps (not including AUG coding for methionine). We calculate that the approximately 53% proteinase K resistance (Figure 3.4A) treated as a total product yield of a 5-step synthesis would imply an 88% yield for each step corresponding to *N*-methyl-Phe incorporation at GUA as compared to the natural amino acid.

In another titration experiment we varied the ratio of NMF-tRNA^{UAC} to either Val-tRNA^{UAC} (Figure 3.4B) or Phe-tRNA^{UAC} (Figure 3.4C) in 5G template translation reactions while keeping the total tRNA^{UAC} concentration constant. As the concentration of NMF-tRNA^{UAC} was decreased relative to the concentration of Val- or Phe-tRNA^{UAC}, the amount of fusion products resistant to digestion by proteinase K were reduced below the 41P control. This general trend suggests that the levels of chemically-acylated tRNAs drive competition for amino acid insertion in our system. Additionally, this data (Figure 3.4C) confirms that proteolysis resistance is indeed a function of *N*-methyl substitution on phenylalanine and not a product of suppression per se. We repeated this experiment in commercial rabbit reticulocyte lysate (Figure 3.5), yielding similar results. This is not surprising since a previous selection demonstrated that tRNAs recognizing the GUA codon in rabbit reticulocyte lysate can be readily suppressed prior to ethanolamine-Sepharose chromatography (*11*).

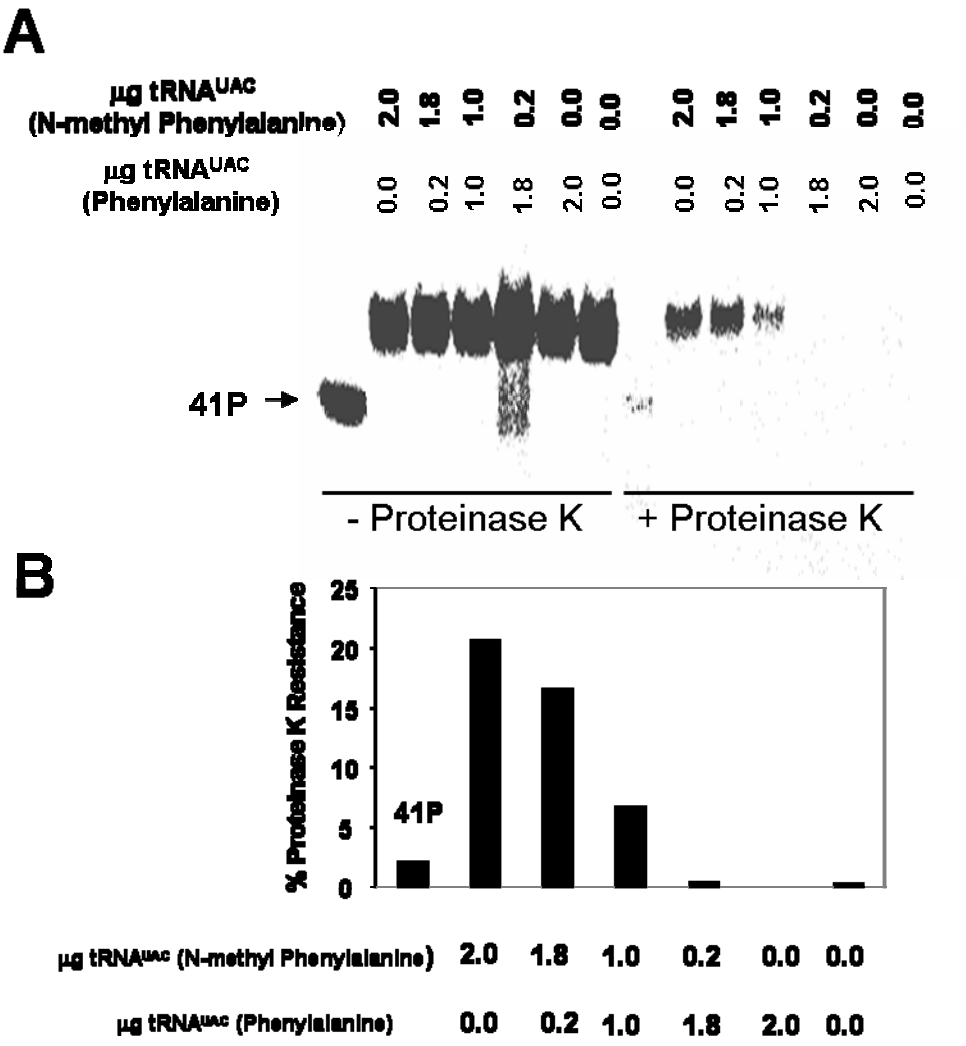

Figure 3.5: Titration of NMF-tRNA^{UAC} and Phe-tRNA^{UAC} in commercial RRL. The 5G template was translated in commercial rabbit reticulocyte lysate (RRL) in the presence of indicated amounts of NMF-Phe-tRNA^{UAC} and Phe-tRNA^{UAC}. (A) Purified ^{35}S-labeled mRNA-peptide fusions (6 µL) were treated with or without 10 µg proteinase K for 60 min at 37 ˚C as indicated and subsequently run on a 10% Tricine-PAGE gel for phosphorimaging. The 41P size control is indicated by the arrow. (B) The fractional resistance to proteinase K degradation was determined by phosphorimaging.

To determine the effect on proteolysis resistance of a single natural amino acid insertion into the middle of an *N*-methyl-Phe encodamer, we designed the 5G* template containing a single GCU alanine codon flanked at either side by two GUA codons. As illustrated in Figure 3.6A, translation without chemically-acylated tRNAs–essentially leaving codons "open" for natural amino acid insertion–would presumably result in the $Met(Val)_2Ala(Val)_2$ fusion. If *N*-methyl-Phe-$tRNA^{UAC}$ is used, however, GUA codons should program the insertion of the unnatural amino acid, leaving GCU to code for Ala. This gives us an opportunity to explore the effect a single amino acid at the GCU position can have on the proteolytic stability of the translated 5G* encodamer by suppressing GCU using different chemically-acylated $tRNA^{AGC}$s. We find that the site-specific incorporation of *N*-methyl-Phe at GCU results in a 1.3-fold greater stability over the "open" codon, and a 2.6-fold greater stability over either Val or Phe (Figure 3.6B). These results indicate the relative enhancement in proteolysis resistance for *N*-methyl-Phe incorporation at a single position within the context of a full-length encodamer.

N-methyl-Phe monomers contain substituents at the α-carbon and the α-*N*-substituted amine that together provide novel structural diversity. The anomalous solubility characteristics of phenylalanine homopolymers have been well established, and proved useful for Nirenberg and Matthaei almost half a century ago when they discovered that poly(U) codes for poly(L-Phe) (*24*). We have shown that the longer 10G *N*-methyl-Phe encodamer aggregates on Urea-PAGE, whereas the shorter encodamers migrate within the gel matrix (Figure 3.2). In addition to removing H-bond donors from the main chain, appending a methyl group on the amino-nitrogen confers proteolytic stability for the encoded peptides. It is an open question as to whether the incorporation of N-methylated

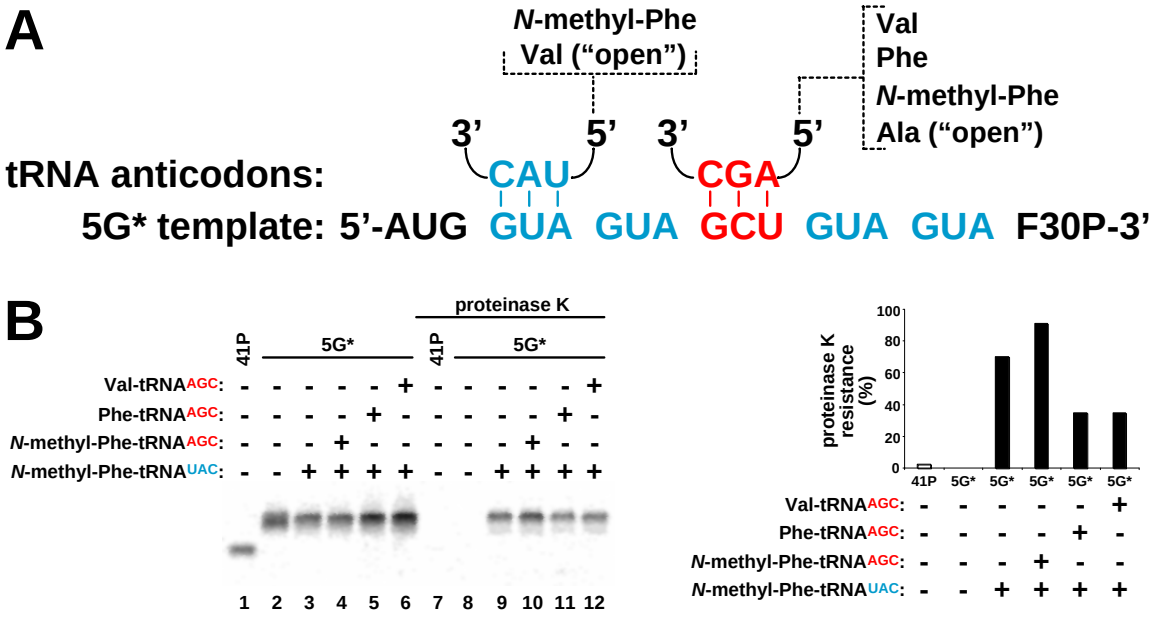

Figure 3.6: Site-specific amino acid incorporation within *N*-methyl-Phe encodamers. (A) The 5G* template, which contains a single GCU codon flanked by GUA codons, can be translated in the presence or absence of various chemically-acylated tRNAs. Codon-anticodon pairs in blue represent suppression of GUA valine, and codon-anticodon pairs in red represent suppression of GCU alanine. (B) The 5G* template was translated in the presence of 2 µg each chemically-acylated tRNA indicated. Purified 35S-labeled mRNA-peptide fusions (10 µL) were treated with or without 10 µg proteinase K for 60 min at 37 ˚C as indicated and run on a 10% Tricine-PAGE gel for phosphorimaging. The ratios of stable encodamers after proteinase K treatment (lanes 7-12) to the untreated encodamers (lanes 1-6) were determined by phosphorimaging and are shown in the graph (right).

amino acids will confer similar properties to other peptide sequences. The approach applied here to N-methyl-Phe polymers may also be applied to other interesting monomer classes such as the N-substituted glycines. Similar to the *N*-methyl-Phe polymers made in this study, oligomers of *N*-substituted glycines (NSGs) or "peptoids" contain residues on their amino nitrogens rather than at the α-carbon positions (*25*). *N*-alkyl glycine libraries are particularly attractive for selection of therapeutic compounds for several reasons, including protease resistance (*26*), the potential for defined secondary structure in solution, and high cellular permeability (*27-30*). However, synthetic peptoid-based libraries are inherently limited in diversity due to the absence of a functional group at the α-carbon and the difficulty in characterizing the products of a selection.

The translation machinery is geared towards accurate polymer synthesis and presents the advantages of multiple-site incorporation of a diverse monomer set for encoded combinatorial libraries comprised of unnatural oligomers. The ribosome can accommodate a wide range of unnatural amino acids, including those with novel side chains (*31-33*) or altered connectivities (*34-40*). Chamberlin and co-workers were the first to incorporate *N*-methyl phenylalanine via nonsense suppression into peptides translated in rabbit reticulocyte lysate (*34*). Surprisingly, this residue was found to have remarkably high translation efficiency (91% relative to glycine) despite the reduced nucleophilicity of the secondary Cα amine. We have determined that the step-wise yield for *N*-methyl phenylalanine incorporation in the 5G template is similar (88%) to that found using nonsense suppression. Unlike nonsense suppression, which is limited to the addition of a 21st amino acid, suppression at sense codons enables the incorporation of a larger number of unnatural monomers into the peptide chain.

Fully reconstituted *Escherichia coli* translation systems have recently been developed to create novel peptides and proteins using chemically-acylated tRNAs to suppress nonsense (*41*) and sense codons (*14*, *42*). These systems, however, have yet to create encoded combinatorial libraries. In our eukaryotic translation system ribosome-mediated synthesis of unnatural oligomers with dramatically reduced susceptibility to proteolytic enzymes can readily be encoded within an mRNA display library.

Materials and Methods

Syntheses

The coupling of *N*-protected phenylalanine derivatives and other amino acids to pdCpA has been described previously (*34*, *43*, *44*) and is outlined below with some minor modifications.

Synthesis of *N*-methyl, *N*-nitroveratrylcarbonyl phenylalanine cyanomethyl ester

Approximately 100 mg *N*-methyl-L-phenylalanine (558 μmol) was dissolved in 2 mL 10% $NaCO_3$ and 1 mL dioxane and cooled to 4 °C in an ice bath. To this, 154 mg (558 μmol) 4,5-dimethoxy-2-nitrobenzyl chloroformate (NVOC-Cl) was added in 3.2 mL dioxane:THF (1:1). The reaction was stirred at 0 °C for 1 hr and then 25 °C for 3 hr.

The reaction mixture was poured into 30 mL water and extracted twice with 10 mL diethyl ether. The aqueous layer was acidified with concentrated HCl (to pH = 2) and then extracted twice with ethyl acetate. The organic layer was dried over $MgSO_4$ and concentrated by rotary evaporation.

The crude product was dissolved in 3 mL dry DMF and 1.5 mL chloroacetonitrile. To this, 1.9 mL (13.4 mmol) TEA was added and the reaction was stirred under nitrogen for 24 hr at 25 °C. Unreacted chloroacetonitrile and solvent were removed by rotary

evaporation and the product was purified by silica gel chromatography in 95:5 CH_2Cl_2:MeOH. (R_f = 0.7). Product was obtained as a yellow oil. Yield = 73% (185 mg). Analysis by low resolution ESI-MS: expected $[M+Na]^+$ = 480.4; observed $[M+Na]^+$ = 480.2.

Synthesis of *N*-nitroveratrylcarbonyl phenylalanine cyanomethyl ester

Approximately 50 mg (300 µmol) of L-phenylalanine was dissolved in 7.5 mL 35 mM Na_2CO_3 + 5 mL THF. To this was added 82.5 mg (300 µmol) 4,5-dimethoxy-2-nitrobenzyl chloroformate in 5 mL THF. The reaction mixture was stirred at 25 °C for 2 hr then concentrated by rotary evaporation. The crude product was then dissolved in 1.5 mL dry DMF and 1.5 mL chloroacetonitrile. To this was added 1 mL (7.2 mmol) of dry TEA. The reaction mixture was stirred for 24 hr under nitrogen at 25 °C. The reaction was concentrated under reduced pressure and the desired product was purified by silica gel chromatography in 95:5 CH_2Cl_2:EtOAc. (R_f = 0.4). Product was obtained as a yellow solid. Yield = 45% (54 mg). Analysis by low resolution ESI-MS: expected $[M+Na]^+$ = 466.13; observed $[M+Na]^+$ = 466.2.

Synthesis of *N*-methyl, *N*-nitroveratrylcarbonyl phenylalanine-dCA

Commercially synthesized pdCpA was dissolved in 0.01 M tetrabutylammonium hydroxide and allowed to stand at 25 °C for 4 hr, then lyophilized to dryness. 8 µmol of lyophilized pdCpA was transferred to a dry round bottom flask and 18.3 mg (40 µmol) *N*-methyl, *N*-nitroveratrylcarbonyl phenylalanine cyanomethyl ester was added. The solid reagents were dissolved in 400 µL dry DMF and a catalytic amount of tetrabutylammonium acetate was added. The reaction was stirred under nitrogen for 5 hr at 25 °C.

The final product was purified by C18 semi-preparative HPLC: Solvent A = 25 mM NH_4OAC (pH = 4.5):CH_3CN (95:5); Solvent B = 25 mM NH_4OAC (pH = 4.5):CH_3CN (10:90); Gradient = 0-100% B in 60 min; Flow = 5 mL/min; Retention time = 22 min. The fractions containing the product were lyophilized to dryness. The lyophilized product was re-dissolved in 10 mL of 10 mM acetic acid and lyophilized again. Yield = 7.5% after HPLC purification. Analysis by low resolution ESI-MS: expected $[M-H]^-$ = 1035.2; observed $[M-H]^-$ = 1035.0.

Synthesis of *N*-nitroveratrylcarbonyl phenylalanine-dCA

Reaction and purification were performed as described for the synthesis of *N*-methyl, *N*-nitroveratrylcarbonyl phenylalanine-dCA. Yield = 2.5% after HPLC purification. Analysis by low resolution ESI-MS: expected $[M-H]^-$ = 1021.2; observed $[M-H]^-$ = 1021.4.

Synthesis of *N*-nitroveratrylcarbonyl valine-dCA

Reaction and purification were performed as described for the synthesis of *N*-methyl, *N*-nitroveratrylcarbonyl phenylalanine-dCA. Yield = 2.2% after HPLC purification. Analysis by low resolution ESI-MS: expected $[M-H]^-$ = 973.2; observed $[M-H]^-$ = 973.4.

Preparation of Aminoacylated tRNAs

The tRNAs used in this study are based on the THG73 amber suppressor tRNA (*45*), but they have been mutated to contain different anticodon triplets and were prepared as previously described (*11*). Purified tRNAs were ligated to a molar excess of NVOC-protected amino acid-dCA conjugates with T4 RNA ligase (New England Biolabs). Reaction mixtures were extracted in an equal volume of phenol:$CHCl_3$:isoamyl alcohol (25:21:1, pH 5.0), and precipitated with 2.5 volumes ethanol (-20˚C). After drying, the

pellet was resuspended in 1.0 mM sodium acetate, pH 5.2 and adjusted to approximately 40 μM (1.0 mg/mL) for each acylated tRNA (ε_{260} = 750000 M^{-1} cm^{-1}). Before adding to translation reactions, tRNAs were deprotected by a xenon lamp outfitted with a 315 nm cut-off filter for 5 min to remove the NVOC group.

Generation of mRNA-peptide Fusions

Synthetic RNA/DNA hybrid templates were made by the California Institute of Technology DNA Synthesis Facility. They include 41P (5'-GGAGGACGAA AUG-F30P-3'), 2G (5'-GGAGGACGAA AUGGUAGUA-F30P-3'), 5G (5'-GGAGGACGAA AUGGUAGUAG UAGUAGUA-F30P-3'), 10G (5'-GGAGGACGAA AUGGUAGUAG UAGUAGUAGU AGUAGUAGUA GUA-F30P-3'), and 5G* (5'-GGAGGACGAA AUGGUAGUAG CUGUAGUA-F30P-3'), where F30P (5'-$dA_{21}[C_9]_3$dAdCdCP; C_9 = triethylene glycol phosphate, and P = CPG-puromycin, Glen Research) serves as a flexible DNA linker. Templates were gel-purified and desalted by ethanol precipitation, and subsequently 10 pmol of material was translated with 20 μL tRNA-depleted rabbit reticulocyte lysate (*23*) in the presence or absence of 80 pmol (2 μg) aminoacylated-tRNA (typically) at 30 ˚C for 60 min. The translation reactions also contained 250 ng $tRNA_i^{Met}$ (*46*), 1 μL 200 mM creatine phosphate, 2 μL ^{35}S-methionine (10 mCi/mL; 1175.0 Ci/mmol; PerkinElmer Life Sciences, Inc.), and 1 μL SUPERasin (Ambion) in a total volume of 30 μL. It should be noted that amino acid supplementation in translation reactions was limited to ^{35}S-methionine only. mRNA-peptide fusion formation was stimulated by the addition of $MgCl_2$ and KCl to 50 mM and 0.6 M, respectively, prior to overnight incubation at -20 ˚C.

The mRNA-peptide fusions were initially isolated from translation reactions by dT_{25}-cellulose (Pierce) binding in 5 mL isolation buffer (1M NaCl, 100 mM Tris-HCl pH 8.0, 0.2% Triton X-100) at 4 ˚C for 45 min, washed in 700 µL isolation buffer seven times at 4 ˚C, and eluted in 500 µL water (ambient temperature). Purified mRNA-peptide fusions were concentrated via ethanol precipitation in the presence of 30 µg linear acrylamide (Ambion) and resuspended in the necessary volume of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to achieve approximately 4000 cpm per 10 µL.

References

(1) Weiss, G. A., Watanabe, C. K., Zhong, A., Goddard, A., and Sidhu, S. S. (2000) Rapid mapping of protein functional epitopes by combinatorial alanine scanning. *Proc Natl Acad Sci U S A 97*, 8950-4.

(2) Takahashi, T. T., Austin, R. J., and Roberts, R. W. (2003) mRNA display: ligand discovery, interaction analysis and beyond. *Trends Biochem Sci 28*, 159-65.

(3) Aina, O. H., Sroka, T. C., Chen, M. L., and Lam, K. S. (2002) Therapeutic cancer targeting peptides. *Biopolymers 66*, 184-99.

(4) Burkoth, T. S., Beausoleil, E., Kaur, S., Tang, D., Cohen, F. E., and Zuckermann, R. N. (2002) Toward the synthesis of artificial proteins: the discovery of an amphiphilic helical peptoid assembly. *Chem Biol 9*, 647-54.

(5) Hanes, J., and Pluckthun, A. (1997) In vitro selection and evolution of functional proteins by using ribosome display. *Proc Natl Acad Sci U S A 94*, 4937-42.

(6) Mattheakis, L. C., Bhatt, R. R., and Dower, W. J. (1994) An in vitro polysome display system for identifying ligands from very large peptide libraries. *Proc Natl Acad Sci U S A 91*, 9022-6.

(7) Merryman, C., Weinstein, E., Wnuk, S. F., and Bartel, D. P. (2002) A bifunctional tRNA for in vitro selection. *Chem Biol 9*, 741-6.

(8) Roberts, R. W., and Szostak, J. W. (1997) RNA-peptide fusions for the in vitro selection of peptides and proteins. *Proc Natl Acad Sci U S A 94*, 12297-302.

(9) Frankel, A., Li, S., Starck, S. R., and Roberts, R. W. (2003) Unnatural RNA display libraries. *Curr Opin Struct Biol 13*, 506-12.

(10) Li, S., Millward, S., and Roberts, R. (2002) In vitro selection of mRNA display libraries containing an unnatural amino acid. *J Am Chem Soc 124*, 9972-3.

(11) Frankel, A., and Roberts, R. W. (2003) In vitro selection for sense codon suppression. *RNA 9*, 780-6.

(12) Li, S., and Roberts, R. W. (2003) A novel strategy for in vitro selection of peptide-drug conjugates. *Chem Biol 10*, 233-9.

(13) Hohsaka, T., Ashizuka, Y., Sasaki, H., Murakami, H., and Sisido, M. (1999) Incorporation of Two Different Nonnatural Amino Acids Independently into a Single Protein through Extension of the Genetic Code. *J Am Chem Soc 121*, 12194-12195.

(14) Forster, A. C., Tan, Z., Nalam, M. N., Lin, H., Qu, H., Cornish, V. W., and Blacklow, S. C. (2003) Programming peptidomimetic syntheses by translating genetic codes designed de novo. *Proc Natl Acad Sci U S A 100*, 6353-7.

(15) Cane, D. E., Walsh, C. T., and Khosla, C. (1998) Harnessing the biosynthetic code: combinations, permutations, and mutations. *Science 282*, 63-8.

(16) Walsh, C. T., Chen, H., Keating, T. A., Hubbard, B. K., Losey, H. C., Luo, L., Marshall, C. G., Miller, D. A., and Patel, H. M. (2001) Tailoring enzymes that modify nonribosomal peptides during and after chain elongation on NRPS assembly lines. *Curr Opin Chem Biol 5*, 525-34.

(17) Lipinski, C. L., F, Dominy, BW; Feeney, PJ. (1997) Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. *Advanced Drug Delivery Reviews 23*, 3-25.

(18) Owens, J. (2003) Chris Lipinski discusses life and chemistry after the Rule of Five. *Drug Discov Today 8*, 12-6.

(19) Goodman, L. S. G., A.; HArdman, J.G.; Gilman, A.G.; Limbird, L.E. (1996) *Goodman and Gilman's Pharmacological Basis of Therpeutics, Ninth Edition*. (McGraw Hill).

(20) Fliri, H., Baumann, G., Enz, A., Kallen, J., Luyten, M., Mikol, V., Movva, R., Quesniaux, V., Schreier, M., Walkinshaw, M., et al. (1993) Cyclosporins. Structure-activity relationships. *Ann N Y Acad Sci 696*, 47-53.

(21) Christians, U., and Sewing, K. F. (1993) Cyclosporin metabolism in transplant patients. *Pharmacol Ther 57*, 291-345.

(22) Fahr, A. (1993) Cyclosporin clinical pharmacokinetics. *Clin Pharmacokinet 24*, 472-95.

(23) Jackson, R. J., Napthine, S., and Brierley, I. (2001) Development of a tRNA-dependent in vitro translation system. *RNA7*, 765-73.

(24) Nirenberg, M. W., and Matthaei, J. H. (1961) The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribonucleotides. *Proc Natl Acad Sci U S A 47*, 1588-602.

(25) Simon, R. J., Kania, R. S., Zuckermann, R. N., Huebner, V. D., Jewell, D. A., Banville, S., Ng, S., Wang, L., Rosenberg, S., Marlowe, C. K., et al. (1992) Peptoids: a modular approach to drug discovery. *Proc Natl Acad Sci U S A 89*, 9367-71.

(26) Miller, S. M. S., R.J.; Ng, S.; Zuckermann, R.N.; Kerr, J.M.; Moos, W.H. (1995) Comparison of the proteolytic susceptibilities of homologous L-amino acid, D-

amino acid, and N-substituted glycine peptide and peptoid oligomers. *Drug Development Research 35*, 20-32.

(27) Kirshenbaum, K., Barron, A. E., Goldsmith, R. A., Armand, P., Bradley, E. K., Truong, K. T., Dill, K. A., Cohen, F. E., and Zuckermann, R. N. (1998) Sequence-specific polypeptoids: a diverse family of heteropolymers with stable secondary structure. *Proc Natl Acad Sci U S A 95*, 4303-8.

(28) Nguyen, J. T., Porter, M., Amoui, M., Miller, W. T., Zuckermann, R. N., and Lim, W. A. (2000) Improving SH3 domain ligand selectivity using a non-natural scaffold. *Chem Biol 7*, 463-73.

(29) Nguyen, J. T., Turck, C. W., Cohen, F. E., Zuckermann, R. N., and Lim, W. A. (1998) Exploiting the basis of proline recognition by SH3 and WW domains: design of N-substituted inhibitors. *Science 282*, 2088-92.

(30) Wender, P. A., Mitchell, D. J., Pattabiraman, K., Pelkey, E. T., Steinman, L., and Rothbard, J. B. (2000) The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proc Natl Acad Sci U S A 97*, 13003-8.

(31) Cornish, V. W. M., D.; Schultz, P.G. (1995) Probing Protein Structure and Function With an Expanded Genetic Code. *Angew Chem Int Ed Engl 34*, 621-633.

(32) Gilmore, M. A., Lance, E. S., and Chamberlin, A. R. (1999) *Incorporation of Noncoded Amino Acids by In Vitro Protein Biosynthesis*.

(33) van Hest, J. C., and Tirrell, D. A. (2001) Protein-based materials, toward a new level of structural control. *Chem Commun (Camb)*, 1897-904.

(34) Bain, J. D., Wacker, D. A., Kuo, E. E., and Chamberlin, A. R. (1991) Site-specific incorporation of non-natural residues into peptides: Effect of residue structure on suppression and translation efficiencies. *Tetrahedron 47*, 2389.

(35) Eisenhauer, B. M., and Hecht, S. M. (2002) Site-specific incorporation of (aminooxy)acetic acid into proteins. *Biochemistry 41*, 11472-8.

(36) Ellman, J. A., Mendel, D., and Schultz, P. G. (1992) Site-specific incorporation of novel backbone structures into proteins. *Science 255*, 197-200.

(37) Killian, J. A., Van Cleve, M. D., Shayo, Y. F., and Hecht, S. M. (1998) Ribosome-Mediated Incorporation of Hydrazinophenylalanine into Modified Peptide and Protein Analogues. *J Am Chem Soc 120*, 3032-3042.

(38) Koh, J. T., Cornish, V. W., and Schultz, P. G. (1997) An experimental approach to evaluating the role of backbone interactions in proteins using unnatural amino acid mutagenesis. *Biochemistry 36*, 11314-22.

(39) Mendel, D., Ellman, J., and Schultz, P. G. (1993) Protein biosynthesis with conformationally restricted amino acids. *J Am Chem Soc 115*, 4359-4360.

(40) Roesser, J. R., Chorghade, M. S., and Hecht, S. M. (1986) Ribosome-catalyzed formation of an abnormal peptide analogue. *Biochemistry 25*, 6361-5.

(41) Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K., and Ueda, T. (2001) Cell-free translation reconstituted with purified components. *Nat Biotechnol 19*, 751-5.

(42) Forster, A. C., Weissbach, H., and Blacklow, S. C. (2001) A simplified reconstitution of mRNA-directed peptide synthesis: activity of the epsilon enhancer and an unnatural amino acid. *Anal Biochem 297*, 60-70.

(43) Ellman, J. M., D.; Anthony-Cahill, S.; Noren, C.J.; Schultz, P.G. (1991) Biosynthetic Method for Introducing Unnatural Amino Acids Site-Specifically Into Proteins. *Methods in Enzymology 202*, 301-336.

(44) Gallivan, J. P., Lester, H. A., and Dougherty, D. A. (1997) Site-specific incorporation of biotinylated amino acids to identify surface-exposed residues in integral membrane proteins. *Chem Biol 4*, 739-49.

(45) Saks, M. E., Sampson, J. R., Nowak, M. W., Kearney, P. C., Du, F., Abelson, J. N., Lester, H. A., and Dougherty, D. A. (1996) An engineered Tetrahymena tRNAGln for in vivo incorporation of unnatural amino acids into proteins by nonsense suppression. *J Biol Chem 271*, 23169-75.

(46) Pestova, T. V., and Hellen, C. U. (2001) Preparation and activity of synthetic unmodified mammalian tRNAi(Met) in initiation of translation in vitro. *RNA 7*, 1496-505.

(47) Schagger, H., and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem 166*, 368-79.