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Encodamers: Unnatural Peptide Oligomers Encoded in RNA

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Summary

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 protein synthesis 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 an *N*-substituted amino acid 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 outside the universal genetic code.

Introduction

The development of therapeutic peptide ligands is an important application of combinatorial libraries. Traditionally, peptide ligand discovery has been explored in two markedly 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 proteolysis 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 in mRNA display libraries by nonsense suppression [10] and sense suppression [11] at single sites as well as site specifically by 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 four 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 (2–12 residues) would facilitate construction and selection of molecules similar to therapeutically important natural products produced by nonribosomal 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. Nonribosomal 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"; molecular weight, 1203.6 g $\times$ 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 two, five, or ten 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 1).

Results and Discussion

Our initial work started with generating polymers of unnatural amino acids encoded as mRNA-peptide fusions.

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Table 1. Reassignment of Codons in This Study

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

This effort requires a subversion of the genetic code to include unnatural amino acids via codon reassignment [9]. Previously, we demonstrated efficient sense codon-mediated incorporation of the unnatural residue 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 a rabbit reticulocyte lysate that was 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 chose this lysate as the translation platform to examine creation of mRNA-peptide fusions containing multiple unnatural amino acid insertions.

We designed three templates containing two, five, or ten consecutive GUA valine codons attached to a flexible DNA linker ending with a 3′-puromycin (Figures 1A and 1B). Translation reactions were then performed with each of these templates in the depleted extract in the presence or absence of an *N*-methyl-phenylalanine-$tRNA^{UAC}$, as shown in Figure 1, with the intention of producing Met(*N*-methyl-Phe)$_n$ polymers covalently attached through 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 2A and 2B indicate that translation products are formed both in the presence and absence of the exogenous chemically acylated tRNA. When fusion products are analyzed by denaturing urea-PAGE, mobility differences should reflect any variation in the translated peptide, as 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 *N*-methyl-Phe $tRNA^{UAC}$ 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. This product is distinct from the reaction lacking *N*-methyl-Phe $tRNA^{UAC}$ (lane 6) and consistent with aggregation in the longer hydrophobic *N*-methyl-Phe oligomer.

Analysis of the same *N*-methyl-Phe $tRNA^{UAC}$ 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 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 the chemically acylated tRNA is omitted, a higher mobility series of bands are observed, consistent with synthesis of truncated or valine-containing oligomers.

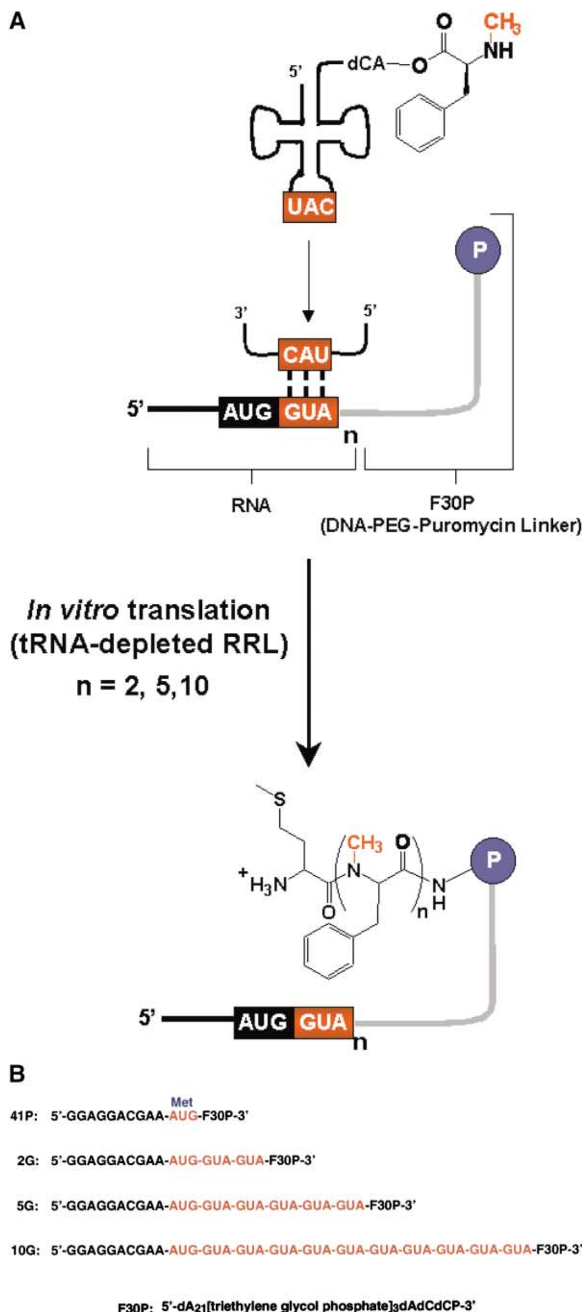

Figure 1. Suppression Scheme and Templates Used

(A) Scheme for inserting *N*-methyl-phenylalanine into mRNA display templates at GUA valine codons. The templates are RNA (black) and DNA (gray) chimeras containing the drug puromycin (P) at the 3′ end. The unnatural amino acid is escorted to the ribosome via a chemically acyleted suppressor $tRNA^{UAC}$.

(B) Templates used to examine unnatural incorporation containing two (2G), five (5G), or ten (10G) consecutive GUA valine codons. The control template 41P containing a single AUG methionine codon is also shown.

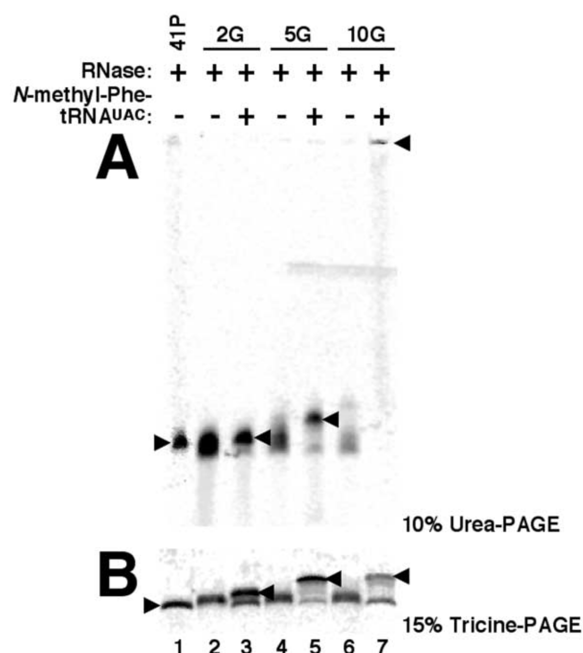

Figure 2. tRNA-Dependence for Full-Length Encodamer Formation

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.

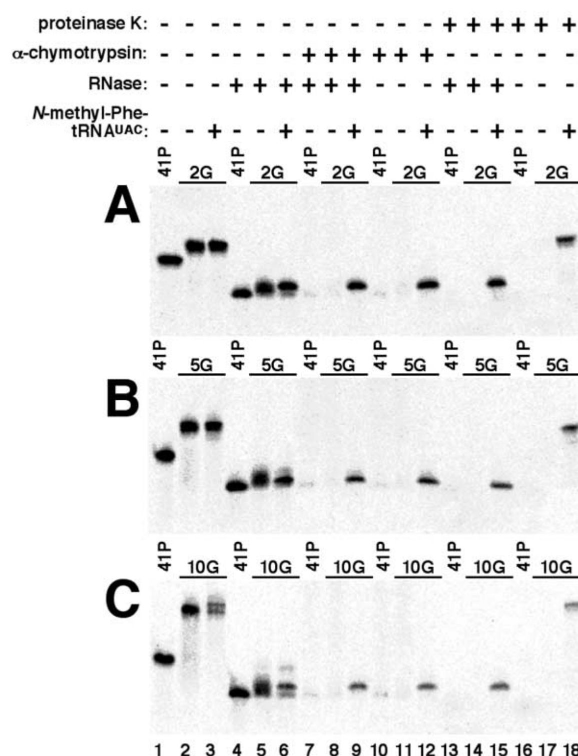

Figure 3. Analysis of Encodamer Resistance to Proteolysis

Templates containing GUA repeats were translated in the presence or absence of 2 μg *N*-methyl-phenylalanine-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)–(C) are phosphorimages of 10% Tricine-PAGE gels for templates 2G, 5G, and 10G, respectively. 41P is shown as a size control.

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. Previously, we had shown that proteinase K efficiently degrades natural mRNA peptide fusion products [8]. The inclusion of *N*-methyl-Phe-tRNA^{UAC} in the reactions results in proteolytically resistant fusions (Figure 3). Treatment of these encodamers with chymotrypsin (apparently containing RNase activity as purchased; Figure 3, lanes 4–12) generates the unnatural oligomer attached to its DNA linker, while treatment with proteinase K (Figure 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 *N*-methyl-Phe-tRNA^{UAC}. It should be noted that the total amount of radiolabeled fusion material decreases in all samples treated with a proteolytic enzyme (Figures 3A–3C, 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 3B and 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 systematically varied the amount of *N*-methyl-Phe-tRNA^{UAC} used in the translation of the 5G template. As shown in Figure 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 *N*-methyl-Phe-tRNA^{UAC} and demonstrates that, at higher concentrations of exogenous tRNA, insertion of the unnatural amino acid outcompetes 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 4A) treated as a total product yield of a five-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 *N*-methyl-Phe-tRNA^{UAC} to either Val-tRNA^{UAC} (Figure 4B) or Phe-tRNA^{UAC} (Figure 4C) in 5G template translation reactions, while keeping the total tRNA^{UAC} concentration

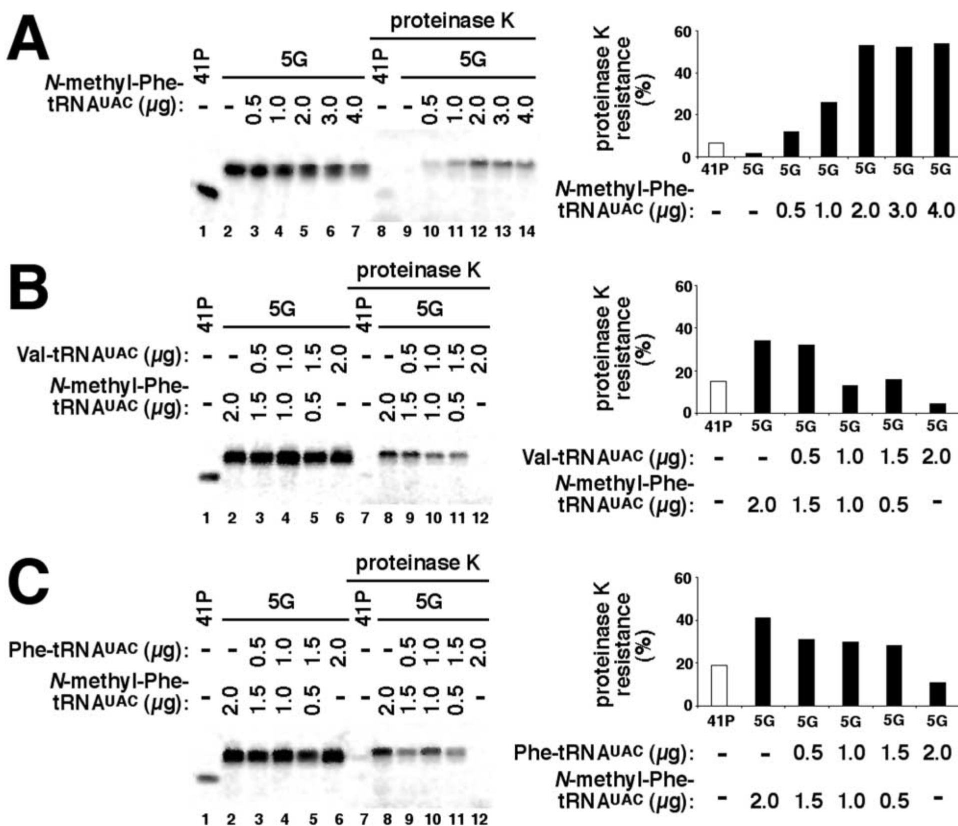

Figure 4. Proteolytic Analysis of *N*-Methyl-Phe Incorporation

(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).

constant. As the concentration of *N*-methyl-Phe-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 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, yielding similar results (data not shown). This is not surprising, since a previous selection demonstrated that tRNAs recognizing the GUA codon in rabbit reticulocyte lysate can be readily suppressed even without ethanolamine-Sepharose chromatography treatment [11].

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 5A, translation without chemically acylated tRNAs—essentially leaving codons "open" for natural amino acid insertion—would presumably result in the Met(Val)$_2$Ala (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 with 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 5B). 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 enco-

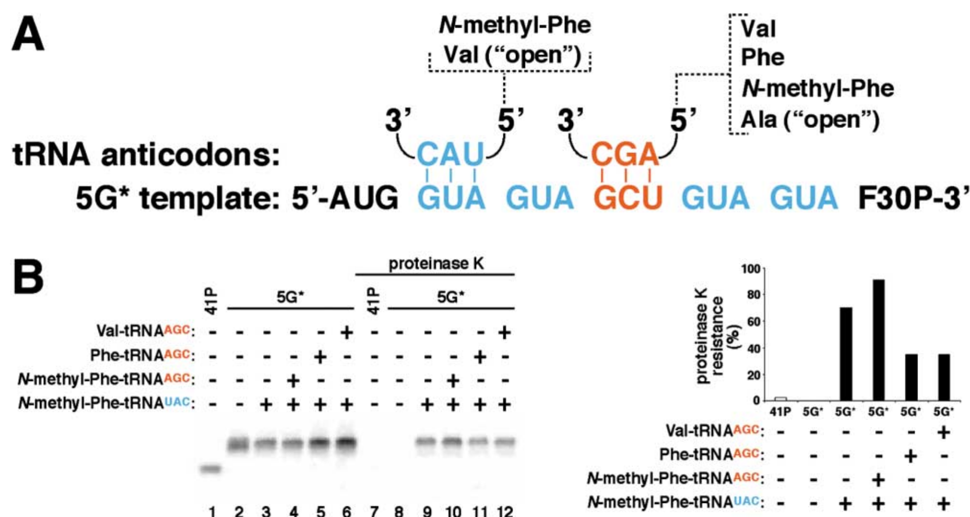

Figure 5. Analysis of Encodamer Synthesis on Nonrepetitive Templates

(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 [^{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 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).

damer aggregates on Urea-PAGE despite being linked to the charged F30P linker, whereas the shorter encodamers migrate within the gel matrix (Figure 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 in line with previous observations in the NRPs. The generality of this result remains to be examined systematically across a great variety of sequences. The approach applied here to *N*-methyl-Phe polymers may also be examined with other interesting classes of monomers, such as the *N*-substituted glycines (NSGs) or "peptoids" that contain side chains appended to their amide nitrogens rather than at the α-carbon position [25]. The peptoid class has some attractive properties, including proteolytic resistance [26], the potential for defined secondary structure, and cell permeability [27–30].

The translation machinery is geared toward 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 coworkers were the first to incorporate *N*-methyl phenylalanine via nonsense suppression into peptides translated in rabbit reticulocyte lysate [35]. 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 stepwise yield for *N*-methyl phenylalanine incorporation in the 5G template is similar (88%) to that found with nonsense suppression. Unlike nonsense suppression, which is limited to the addition of a 21st amino acid to the genetic code, suppression at sense codons allows for a larger number of unnatural monomers that can be incorporated into a peptide chain.

Fully reconstituted *Escherichia coli* translation systems have recently been developed to create novel peptides and proteins with 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 a mRNA display library.

Significance

Libraries generated by mRNA display can contain more than 10^{13} unique molecules from which the sequences of selected molecules can be easily extracted. Here, we have extended the complexity of mRNA display libraries beyond the 20 naturally occurring amino acids by subverting the genetic code to include *N*-methyl-phenylalanine and via codon reassignment of valine and alanine. This system is amenable to creating completely unnatural polymers or encodamers drawing from the diversity of natural and unnatural amino acids. We demonstrate that the orthogonally encoded *N*-methyl-Phe oligomers translated via mRNA display are resistant to proteolysis, a useful property that should enhance the stability of high-affinity in vitro-selected compounds in an in vivo setting. The encodamers described in this study thus mark an initial step in developing more drug-like compounds from encoded combinatorial display libraries.

Experimental Procedures

Syntheses

The coupling of *N*-protected phenylalanine derivatives and other amino acids to pdCpA has been described previously [35, 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 chloracetonitrile. 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. Lyophilized pdCpA (8 μmol) was transferred to a dry round-bottom flask, and *N*-methyl, *N*-nitroveratrylcarbonyl phenylalanine cyanomethyl ester (18.3 mg [40 μmol]) 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 semipreparative 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 redissolved in 10 ml of 10 mM acetic acid and lyophilized again. Yield, 7.5% after HPLC purification. The relatively low yield of this and other dCA-bearing amino acids is likely due to a combination of features, including measurement after HPLC purification and the small scale of the reaction. Analysis by low-resolution ESI-MS: expected $[M-H]^-$, 1035.2; observed $[M-H]^-$, 1035.

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 ($\epsilon_{260\ nm}$, 750,000 M^{-1}*cm^{-1}). Before adding to translation reactions, tRNAs were deprotected by a xenon lamp outfitted with a 315 nm cutoff 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 AUGGU AGUA-F30P-3′), 5G (5′-GGAGGACGAA AUGGUAGUAG UAGUA GUA-F30P-3′), 10G (5′-GGAGGACGAA AUGGUAGUAG UAGUAGU AGU AGUAGUAGUA GUA-F30P-3′), and 5G* (5′-GGAGGACGAA AUGGUAGUA<u>G</u> <u>CU</u>GUAGUA-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] prepared as previously described [11] in the presence or absence of 80 pmol (2 μg) aminoacylated-tRNA (typically) at 30°C for 60 min [11]. 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 SUPERase-In (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.

Acknowledgments

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