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Unnatural RNA display libraries

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Combinatorial peptide and protein libraries have now been developed to accommodate unnatural amino acids in a genetically encoded format via *in vitro* nonsense and sense suppression. General translation features and specific regioselective and stereoselective properties of the ribosome endow these libraries with a broad chemical diversity. Alternatively, amino acid residues can be chemically derivatized post-translationally to add preferred functionality to the encoded peptide. All of these efforts are advancing combinatorial peptide and protein libraries for enhanced ligands against biological targets of interest.

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Introduction

The search for novel peptide ligands from diverse combinatorial libraries has resulted in the isolation of specific inhibitors of relevant targets, such as cancer-related proteins [1]. Combinatorial peptide and protein libraries are manufactured either biologically [2–4] or synthetically [5], each having their own advantages and disadvantages. Phage display [2,6], ribosome display [7,8], tRNA display [9] and mRNA display libraries [4,10] are advantageous because they can be easily amplified for iterative cycles of selection as each peptide or protein is associated with its encoding DNA or mRNA sequence. Furthermore, biological libraries often contain peptides that mimic natural ligands for cell surface receptors [11]. By contrast, synthetic libraries are typically orders of magnitude smaller in size and require deconvolution steps [12] or sophisticated encoding/decoding schemes [13] to identify active molecules; however, their complexity is not fettered to the 20 natural amino acids as in biological libraries. The limited diversity and biological instability of natural polypeptides selected from biological libraries make the generation of drug-like compounds very challenging.

In nature, the chemical complexity of amino acid side-chains is extended by various post-translational modifica-

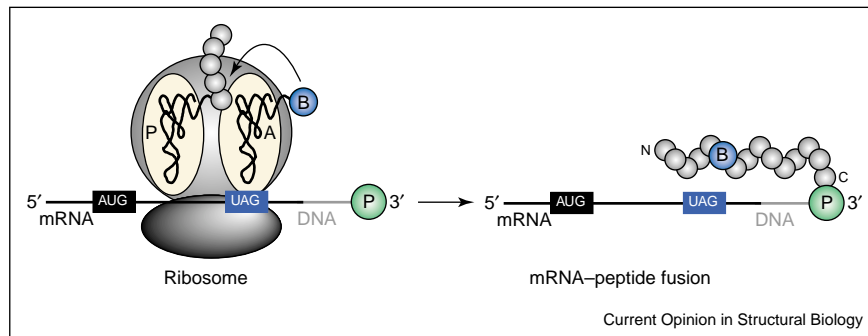
tions essential to the normal function and regulation of many proteins, including glycosylation, phosphorylation, methylation, isoprenylation and acetylation [14]. Chemical complexity can also be achieved via the site-specific incorporation of unnatural amino acids into proteins during translation. In 1989, the Schultz and Chamberlin groups [15,16] demonstrated the insertion of an unnatural amino acid using nonsense codon suppression *in vitro*, by escorting the residue to the ribosome with a chemically charged amber suppressor tRNA. The strategy of nonsense suppression also extends to unnatural amino acid insertion into proteins *in vivo* [17,18] and has been reviewed in detail elsewhere [19,20,21]. This review describes recent advances in unnatural amino acid incorporation into *in vitro* biological display libraries by various methodologies and addresses the chemical plasticity of the translation machinery required for libraries containing unnatural amino acids.

To date, all experiments combining *in vitro* selection with unnatural amino acid incorporation have been performed using mRNA display methods [4,22]. In mRNA display, libraries are generated *in vitro* by translating mRNA molecules bearing a 3' puromycin residue (reviewed in [23]). After translation, the templates become covalently attached to the peptide or protein they encode when the puromycin molecule enters the ribosome and attaches to the C terminus of the nascent peptide chain. The resulting mRNA–peptide or mRNA–protein fusions can be sieved based on protein function, and the encoding sequence amplified and recovered using RT-PCR [4]. This approach enables the construction of peptide and protein libraries containing more than 10^{13} members [22,24,25]. In principle, *in vitro* selection based on unnatural amino acids could also be performed via either tRNA display [9] or ribosome display [7,8] in the future. Phage display [2,6] may also serve as a future vector for unnatural libraries by combining this approach with *in vivo* nonsense suppression [17,18].

Strategies to add a 21st amino acid

Li *et al.* [26] provided the first demonstration that nonsense suppression could be combined with an *in vitro* selection experiment. Biocytin (a biotin derivative of lysine) was incorporated into an mRNA display library [26] and used to select templates that efficiently incorporated the unnatural residue. The biocytin residue was shuttled into the ribosome by a semi-synthetic tRNA, the engineered THG73 amber suppressor tRNA [27] (Figure 1). After two rounds of *in vitro* selection against streptavidin–agarose, templates containing the UAG stop codon dominated the library, representing >85% of the

Figure 1



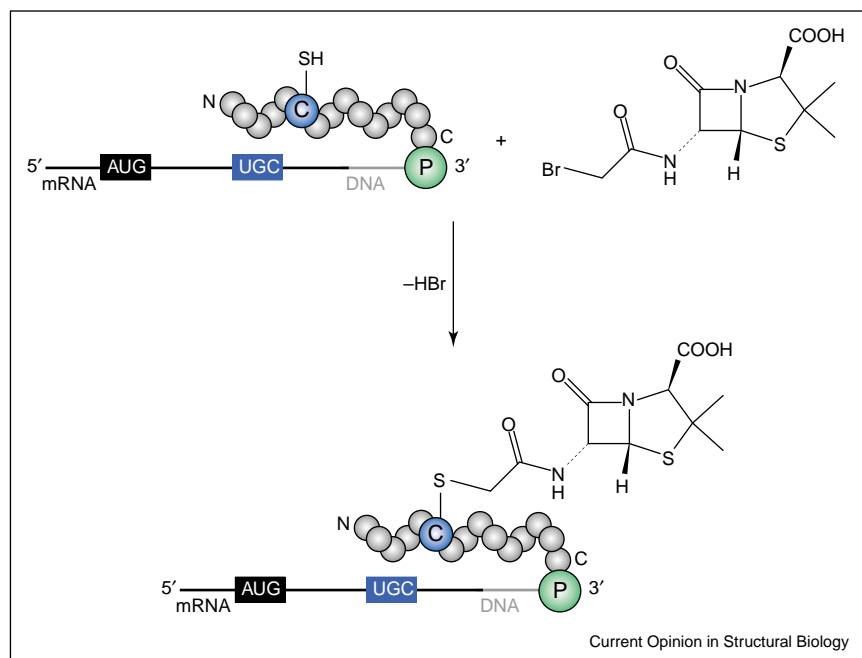
Nonsense suppression in mRNA display. Translation of a template tethered to a 3' puromycin (P) (green) and containing a UAG stop codon within the coding sequence requires an acylated amber suppressor tRNA and results in an mRNA-peptide fusion bearing the unnatural amino acid biocytin (B) (blue).

sequences present. These experiments demonstrated that selection could occur based on an unnatural residue inserted at the UAG blank in the genetic code.

Chemical derivatization provides an alternative to nonsense suppression for inserting a 21st amino acid [28**]. These semi-synthetic libraries provide a flexible means to add diversity, rigidity or other improved properties to peptide and protein libraries. Among the 20 naturally occurring amino acids, cysteine was chosen as it provides a reactive and chemically orthogonal functional group for specific covalent modification of proteins. In this

work, a fixed cysteine residue was modified post-translationally using a bromoacetyl derivative of penicillin (Figure 2). Despite the fact that the fusion templates are typically present at low concentrations (submicromolar), approximately 20% of a 15 trillion member peptide library could be modified with penicillin, resulting in a hybrid peptide-drug library containing three trillion independent conjugates. *In vitro* selection of this library against penicillin-binding protein 2a (PBP2a), the drug-resistant determinant in methicillin-resistant *Staphylococcus aureus* [29], resulted in two ligands with 100-fold higher activity than the parent molecule. This chemical

Figure 2



Post-translational modification of an mRNA-peptide fusion library to contain penicillin. Reacting sodium 6-bromoacetyl penicillin to an mRNA display library containing a fixed cysteine residue (blue) forms a peptide-drug conjugate through a thioether linkage.

derivatization strategy mimics the naturally occurring post-translational modifications found in proteins and provides a means to target display libraries to specific protein surfaces or domains based on the affinity or reactivity of a small molecule.

The nonsense suppression and chemical derivatization strategies provide a bridge between biopolymer libraries and synthetic small molecules, previously considered to be two distinct fields. The incorporation of a small lead compound into an amplifiable library holds the promise for rapid optimization of its affinity and specificity, thus considerably facilitating the drug discovery process.

Unnatural amino acid incorporation at sense codons

Multiple-site incorporation with more than one unnatural amino acid requires a reprogrammed genetic code that may involve partial or ubiquitous reassignment of the 61 sense codons. Substituting the meaning of a codon specific to one amino acid for another during translation is defined as a missense error [30]. These errors occur on the order of 10^{-3} to 10^{-4} , as measured by the incorporation of a noncoded amino acid into a protein, caused either by tRNA misacylation or by productive pairing of a codon with a noncognate anticodon on the ribosome [31,32]. Historically, missense suppression and codon reassignment have been known *in vivo* for some time, and have been well studied in the context of the suppression of *trpA* mutants of *Escherichia coli* [33]. These *trpA* mutants encode inactive tryptophan synthetase A because of crucial amino acid substitutions (missense mutations) at wild-type glycine positions, resulting in tryptophan auxotrophy. Missense suppressors of *trpA* mutants inserted, for example, glycine for arginine at the rare AGA codon [34,35], thus restoring tryptophan prototrophy. These suppressors were determined to be tRNA^{Gly} species mutated within the anticodon to base pair with nonglycine codons [36–38]. *In vivo* missense suppression efficiencies are generally low (e.g. <4% of tryptophan synthetase A), as it is likely that higher levels of missense suppression result in intolerable substitutions in proteins throughout the cell, causing rampant toxicity [33].

In vitro, problems due to the effects of missense suppression on viability are eliminated. Attempts to incorporate unnatural amino acids at rare codons using *in vitro* translation systems have succeeded to varying degrees. Sisido and co-workers [39] reported the use of an *E. coli* S30 translation system to incorporate photofunctional groups into a polypeptide at a single rare AGG arginine codon via a chemically acylated orthogonal tRNA_{CCU}, resulting in polypeptides with slightly altered spectroscopic properties. A fascinating alternative to the *E. coli* S30 translation system is the one prepared from *Micrococcus luteus*, an organism devoid of six codons and their complementary tRNAs [40,41]. Complete translation of *E. coli* genes in

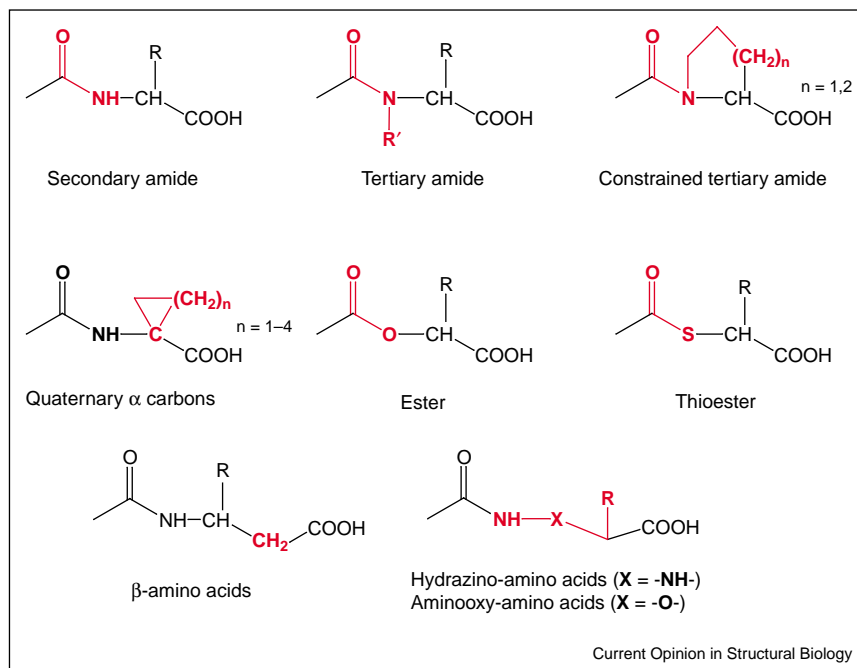
M. luteus extracts is dependent on an exogenous supply of *E. coli* tRNAs that presumably contains complementary tRNAs [42]. In this case, read-through of the missing codon AGA occurred only when a complementary phenylalanine-acylated tRNA was supplied [43]. Together, these early *in vivo* and *in vitro* experiments argue that it should be possible to predict sense codon suppression efficiency, as codon usage and tRNA abundance parallel one another [44].

mRNA display was recently used to probe the 16 GNN codons for positions that lead to efficient incorporation of an unnatural amino acid in a peptide–mRNA fusion [45**]. Briefly, a library containing 64 independent templates was translated in the presence of 16 biocytin-acylated tRNAs, each of which could recognize a GNN codon by exact Watson–Crick pairing. Templates that efficiently incorporated the unnatural residue in response to a sense codon could be enriched on streptavidin, similar to the selection for nonsense suppression shown in Figure 1.

After several rounds of selection for streptavidin binding, the coding template became predominantly enriched with GUA (valine) at the randomized codon position. This level of sense suppression (40–50%) was equal to or better than the corresponding amber suppression. In the commercial lysate, suppression of alanine codons GCG and GCU was much lower (7–20%). Strikingly, insertion at the alanine positions became equivalent to both nonsense and GUA-mediated valine suppression by passing the translation lysate over an ethanolamine–sepharose column. This column protocol depletes the majority of endogenous tRNAs [46]. These results indicate that modest manipulation of a translation lysate provides a platform to use arbitrarily chosen codons for insertion of unnatural residues. In this vein, recently described partially or completely reconstituted translation systems containing purified *E. coli* components [47**,48*] should also facilitate inserting unnatural residues in response to sense and nonsense codons alike. In line with that view, Forster *et al.* [49*] have recently reported the construction of short unnatural oligopeptides in a reconstituted system.

The genetic code as we know it contains 61 sense and 3 nonsense codons. In addition to codon reassignment, noteworthy efforts to expand the genetic code to incorporate unnatural amino acids into nascent polypeptides at original codons have been addressed via novel codon–anticodon interactions, such as unnatural base pairing and four-base codon–anticodon pairing (reviewed elsewhere [20*]). These methods provide the tantalizing attraction of using a much larger set of monomers to create molecular libraries. On the other hand, mixing codons with different lengths makes what peptide a particular template encodes ambiguous, due to uncertainty in the reading frame after the first residue.

Figure 3



Chemical scope of the ribosome. The types of residues and bond-forming reactions possible with prokaryotic and eukaryotic ribosomes.

Chemical scope of the ribosome

The idea of using the ribosome to create unnatural molecular libraries raises the issue of what residues can be joined with reasonable efficiency. The chemical scope of the ribosome has been addressed historically in two general ways: by constructing small-molecule puromycin analogs bearing various pendant amino acids [50], or using chemically or enzymatically acylated tRNAs [51,52]. A summary of the residues and chemistry possible using the ribosome is shown in Figure 3.

Puromycin analogs represent a means to test and quantify the action of amino acids and other residues in the ribosome active site, as these represent a soluble factor-independent means to assess ribosome activity [53,54]. Puromycin derivatives bearing various amino acids demonstrated that the ribosome preferred bulky hydrophobic amino acids to small sidechains and that peptide bond formation was stereospecific for natural [L] amino acids [50]. Subsequently, puromycin derivatives have been used to show that the ribosome can catalyze ester bond formation [55], as well as thioester bond formation [56]. More recently, puromycin derivatives have been used to analyze the stereospecificity and regiospecificity of the ribosome [57••]. The stereoselectivity of the ribosome appears to be a function of sidechain size, with larger residues (e.g. 4-methylphenylalanine and *O*-methyltyrosine) showing higher levels of chiral selectivity in peptide bond formation (2400- and 150-fold, respectively) than

smaller residues such as alanine (~ 3 -fold) [57••]. These experiments were also used to show that β -amino acids (residues containing a three-carbon backbone) could also participate with reasonable efficiency in peptide bond formation. Among the substituted β -amino acids examined, chiral selectivity seems to be modest. However, the efficiency of β -residues bearing chiral centers and sidechains adjacent to the carboxylate is yet to be examined.

The chemical scope of the ribosome has also been explored extensively using aminoacyl-tRNAs (reviewed in [58,59,60••]). These experiments reveal the versatility of the ribosome as a synthetic machine. Among [L] amino acids, the pendant sidechain can vary from being a proton (e.g. glycine) to a long flexible chain (e.g. biocytin) to more rigid aromatic systems [58]. The ribosome is also surprisingly tolerant of several changes in the backbone, including the attacking nucleophile, *N*-methyl and *N*-ethyl substituted residues, residues cyclized between the amine and α -carbons, β -amino acids, and residues containing quaternary branched or cyclic α -carbon substitutions [61–63]. Reactive β -amino acid mimics such as hydrazino-amino acids [64] and (aminoxy)acetic acid [65••] have also been incorporated. There are some limitations to the ribosome's versatility; [D] enantiomers [15,51], γ -amino acids (5-aminovaleryl), dipeptides (glycyl-glycyl) [61] and certain spectroscopic probes [66] are not incorporated efficiently. We note that there is hope for engineering the entry of even recalcitrant residues,

either by engineering elongation factor and tRNA interactions [67,68] to compensate for poor substrate utilization in the active site of the ribosome [57**], or by re-engineering the ribosome itself to enable more efficient incorporation [69,70**].

Conclusions

The expansion of encoded combinatorial libraries to include unnatural amino acids and functionally derivatized sidechains is a field in its infancy that should have an enormous impact on therapeutic and diagnostic ligand discovery. Initial work has demonstrated that suppression of nonsense and sense codons, as well as covalent chemical modifications, provide facile means to enhance the diversity of these libraries. The chemical ability of the ribosome is vast, and provides a diverse palette for the construction of truly new encoded molecules for our examination and functional analysis. The union of ribosomal chemistry with modern synthetic methods in the form of unnatural RNA display libraries promises an exciting future of molecular exploration.

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