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In Vitro Selection of mRNA Display Libraries Containing an Unnatural Amino Acid

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Techniques such as phage[1] and mRNA display[2−4] now make it possible to generate very large peptide and protein libraries and sieve them for functional molecules. Presently, the chemical diversity that may be programmed into these libraries is limited to the 20 naturally occurring amino acid side chains. Over 10 years ago, Schultz and co-workers demonstrated that unnatural amino acids, residues that do not occur in normal proteins, may be inserted into proteins if they are escorted to the ribosome by an orthogonal suppressor tRNA recognizing a stop codon.[5,6] This unnatural strategy allows proteins to be constructed that contain a novel residue at a desired location, enabling insertion of affinity tags,[7] spectroscopic probes,[8] and analogues for detailed mechanistic analysis.[9] This suppression strategy has also been extended to eukaryotic systems both in vitro[10,11] and in vivo.[12,13]

Recently, there has been great interest in extending the unnatural strategy to systems where the new residue may be selected for its function. Here, we demonstrate that in vitro selection experiments can be performed on mRNA display libraries containing the unnatural amino acid biocytin (Figure 1A). In mRNA display, translation extracts are used to generate combinatorial libraries of peptides and proteins covalently fused to their own mRNA via a 3′-puromycin. These libraries are strictly monovalent and provide for the synthesis of more than 10^{13} independent peptide or protein sequences in a selectable format.[14−18]

We began by working to synthesize mRNA−peptide fusions containing an unnatural residue. Biocytin, a biotin derivative of lysine, represented an excellent choice for our target residue as this has been inserted into proteins previously,[7] and the biotin moiety could be readily used to select sequences that have incorporated this amino acid. We normally construct mRNA display libraries in the rabbit reticulocyte translation extract due to the excellent stability of the template in this media and the efficiency of fusion formation. We chose the amber suppressor tRNA THG73 (a modified *Tetrehymena thermophila* Gln tRNA) to insert our unnatural residues by nonsense UAG suppression (Figure 1B) as this construct has high efficiency in eukaryotic translation systems.[12]

We next constructed two templates to test insertion of the unnatural residue. The first template (Pep1) is a control containing all 20 amino acids, but no stop codon, while the second template (Pep2) contains a similar amino acid composition and a single UAG stop codon at the third position (Figure 2A). For Pep2, fusion formation occurs only when the suppressor tRNA is added, consistent with incorporation of biocytin into the Pep2 mRNA−peptide fusion (Figure 2B).

We wished to demonstrate that this approach could be used to select peptides from libraries based on the function of the unnatural residue. To do this, we replaced the TTG codon that encoded the

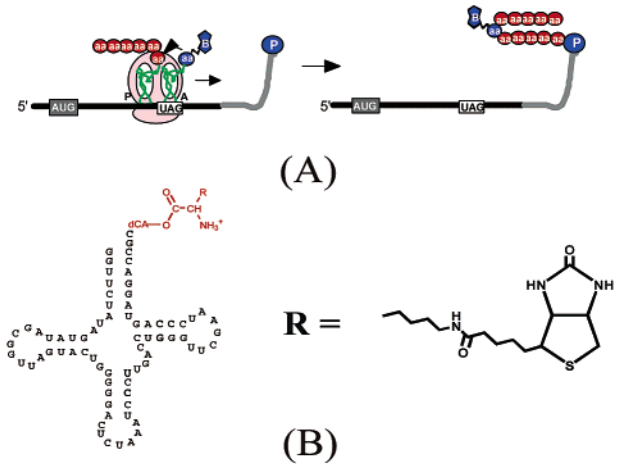

Figure 1. (A) Scheme for insertion of unnatural amino acids into mRNA display libraries via amber suppression. (B) Biocytin-charged THG73.

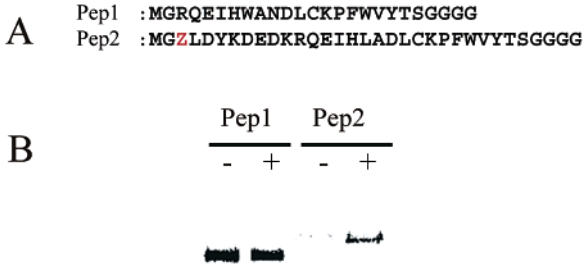

Figure 2. Dependence of fusion formation on addition of biocytin-charged THG73. (A) Sequences of fusion templates tested. (B) Gel showing formation of fusion products, labeled with ^{35}S-methionine, in the presence (+) or absence (−) of biocytin-charged THG73. Fusion formation on the stop codon-containing template (Pep2) occurs only in the presence of the suppressor tRNA.

Trp residue in position 8 of the template pep1 with an NNS saturation cassette containing 32 possible codons encoding all 20 possible amino acids and the UAG stop (Lib1). We then performed two rounds of in vitro selection using streptavidin agarose as our affinity matrix (see Supporting Information for details of the experiments).

Sequencing after one round of selection indicated that UAG stop codons were being enriched at both the randomized position and elsewhere in the open reading frame via point mutations. After a second round of selection versus streptavidin agarose, nine clones were sequenced from the library. Eight out of nine (88%) contain a UAG stop codon at the randomized position or elsewhere, including two that contain a GAG-to-UAG transversion at position 5 (Figure 3).

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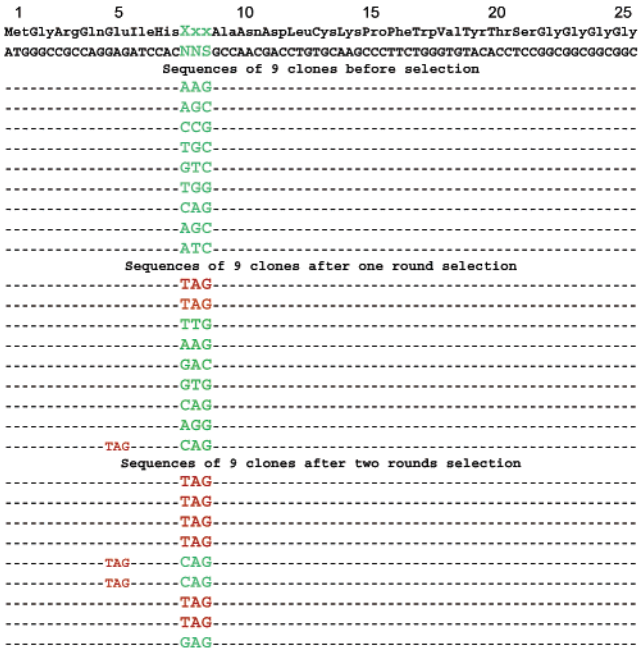

Figure 3. Sequences present in Lib1 before and after selection vs streptavidin agarose. Amino acids sequence in three-letter format of Lib1 before selection is shown in the second line and its DNA sequence is shown in the third line. Xxx represents all 20 amino acid residues plus UAG stop codon. N is equal amount of all four nucleotides, and S is 50% G plus 50% C in that position. The sequences in NNS saturation region are highlighted in green, and the emerging UAG stop codons are highlighted in red. Sequences the same as the original template are labeled with a dash (−).

Our experiments represent the first combination of an in vitro selection experiment and nonsense suppression of which we are aware. Our approach now allows selectable peptide and protein libraries to be constructed containing any nonnatural amino acid that is compatible with the translation apparatus. These libraries should facilitate the discovery of novel ligands with functionalities beyond those provided by the 20 naturally occurring residues. For example, peptides containing N-substituted amino acids are protease-resistant[19] and can show enhanced affinity for natural protein interaction modules such as SH3 domains.[20] Our approach should also aid physical organic dissection of protein interfaces, particularly where molecular interactions display context dependence. Additionally, this approach provides a convenient way to construct new molecular tools based on known pharmacophores, in that therapeutically useful small molecules may be presented adjacent to the chemical diversity present in a 10^{13}-member peptide or protein library. Finally, translation systems that allow insertion of two or more unnatural amino acids[21] now provide the intriguing possibility for construction of wholly unnatural libraries in a selectable mRNA display format.

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Supporting Information Available: Experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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