Point of VIEW
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Unnatural Translation Initiation

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ABSTRACT **Protein translation in nature always begins with an initiator transfer RNA (tRNA) carrying the amino acid methionine. This was circumvented *in vitro* with a reconstituted translation system utilizing initiator tRNA synthetically mischarged with the other natural amino acids. In addition, it was determined that this system could accommodate these non-methionine amino acids containing various *N*-α-acyl groups, many of which are useful for post-translational modification such as peptide cyclization.**

In vitro translation has long been a tool of molecular biologists for the production of proteins and peptides. With the development of genetic code reprogramming, chemists have been playing an increasing role in the progress of this field by moving beyond the limits imposed by the 20 natural amino acid building blocks. The area of translation initiation, however, has remained relatively untouched. To alter the initiation of peptide synthesis, Goto *et al*. (*1*) (p 120 of this issue) integrate two existing technologies, protein synthesis using recombinant elements (PURE) (*2*) and flexizyme (*3*) systems. They were able to efficiently utilize most amino acids (Table 1), rather than the natural methionine residue, for the initiation of translation. With this advancement, both chemists and biologists have a new method in their molecular toolbox for the *in vitro* production of modified peptides and proteins.

The PURE system that the authors used is a cell-free, reconstituted *Escherichia coli* translation system that provides for the production of peptides and proteins encoded on messenger RNA (mRNA). To allow for initiation by amino acids other than methionine, a methionine depletion modification of this system (wPURE) was used. This modification was necessary because the PURE system includes the 20 natural aminoacyl-transfer RNA (tRNA) synthetases (ARSs), which would normally supply the methionyl-loaded initiator tRNA.

Goto *et al*. used the flexizyme system to synthetically load the initiator tRNAs with the various modified amino acids. The flexizyme is a ribozyme that catalyzes the reaction of the desired tRNA with an amino acid synthetically activated as a benzyl ester. This system is amenable to a wide variety of modified and unnatural amino acids, unlike the native ARSs.

After the authors determined the relative initiation efficiencies of the unmodified amino acids, they examined preacylated amino acids. Initiation efficiency was enhanced by the addition of amino acid-initiator tRNA substrates synthetically preacylated with various natural and unnatural *N*-α-acyl groups. This result is especially exciting because it provides access to expression of N-terminally labeled peptides and proteins. The N-terminal labels explored in this work are varied (Table 1). Of special interest are the alkynes and azido functionalities, which would allow the use of "click" chemistry to further modify the peptides.

In addition, the authors determined that incorporation of an *N*-α-acyl group with a reactive chloroacetyl functionality enabled spontaneous peptide cyclization with a cysteine sulfhydryl, yielding a stable thioether linkage. Cyclic peptides are of interest because of their increased stability in biological systems and membrane permeability (*4*). They are more conformationally limited than linear peptides of identical sequence, resulting in a more rigid structure and, potentially, tighter target binding (*5*). Also, many biologically active natural products, such as vancomycin, have a cyclic peptide framework. This method should prove useful in the construction of cyclic peptide libraries. The only other methods for the spontaneous cyclization of proteins and peptides upon translation are those that

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TABLE 1. Comparison of natural and efficient (>50% of natural expression levels) non-natural translation initiators

Initiation	R	R_1
Natural	Formyl	Met
Goto *et al.*	Alkanes, Alkenes, Alkynes, Aryl, Azido, Chloroacetyl	Ala, Val, Ser, Thr, Phe, Tyr, Leu, Ile, Gln, Met, Lys, Trp, His

use inteins, such as those used in the SICLOPPS system (*6, 7*). Alternatively, it is possible to enzymatically effect peptide cyclizations *in vitro* (*8*).

The area for which this research has the greatest potential impact is the production of cyclic peptide libraries. The libraries are easily constructed by incorporation of random nucleotides on the mRNA templates. When combined with the wPURE system and an initiator tRNA loaded with an *N*-α-chloroacetyl amino acid, the library is constrained only by the chosen initiator amino acid and the requirement of having only one cysteine residue for peptide cyclization. Conceivably, this technology could be used to produce peptides with interesting motifs. Rather than head-to-tail cyclizations with the cysteine residue at the C-terminus yielding a simple ring, the cysteine could be placed internally, leading to a peptide with loop and stem regions.

Although the authors produced a 160-member library as a proof of principle, this technology still requires more development before it can be used to generate large, useful peptide libraries. Two additional steps remain to be exploited to maximize this technology's potential. The first step, which the authors acknowledge, would be to combine this system with mRNA display (*9*). This would provide a mechanism for library member identification and *in vitro* screening, both of which are absolute requirements for large (10^{6-14}) libraries. A second improvement would be the incorporation of unnatural amino acids at positions other than the N-terminus to maximize library diversity.

Overall, this work reflects a novel combination of existing technologies that provides facile access (once the systems are established) to natural and unnatural linear and cyclic peptides. The ability to reprogram the translation system and incorporate some N-terminal amino acid modifications lends flexibility in target production that would be difficult to match without the application of extensive synthetic organic chemistry expertise. Although presently useful for the production of small peptide libraries, this system is currently limited for the production of large libraries because the issues of screening and library deconvolution remain. Fortunately, the technique of mRNA display exists and should be easily adapted to this system to overcome this final hurdle.

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