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Pure translation display

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Abstract

Methods such as monoclonal antibody technology, phage display, and ribosome display provide genetic routes to the selection of proteins and peptides with desired properties. However, extension to polymers of unnatural amino acids is problematic because the translation step is always performed in vivo or in crude extracts in the face of competition from natural amino acids. Here, we address this restriction using a pure translation system in which aminoacyl-tRNA synthetases and other competitors are deliberately omitted. First, we show that such a simplified system can synthesize long polypeptides. Second, we demonstrate "pure translation display" by selecting from an mRNA library only those mRNAs that encode a selectable unnatural amino acid upstream of a peptide spacer sequence long enough to span the ribosome tunnel. Pure translation display should enable the directed evolution of peptide analogs with desirable catalytic or pharmacological properties.
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Like earlier technologies [1,2], ribosome display [3] links phenotype to genotype for protein mutagenesis and discovery of polypeptide ligands. This technique made it possible to screen much greater numbers of peptides simultaneously for binding to a target molecule. In ribosome display, a mixture of mRNA transcripts deficient in stop codons is translated by a crude cell extract into stable mRNA/ribosome/peptidyl-tRNA complexes. These complexes are then selected for the ability to bind to any immobilized target molecule. mRNAs are eluted from bound complexes, amplified, and mutated (via reverse transcription/mutagenic PCR), and the resulting DNAs are transcribed and translated for another round of selection. This cycle is reiterated until only DNAs encoding polypeptide ligands remain, and the sequences of the ligands are deduced by sequencing the DNAs that encoded them. This totally cell-free crude translation display was originally proposed [4] as a way to overcome two limitations of screens that require passage through cells (e.g., yeast two-hybrid [5] and phage display [2,6]): the restriction of library size due to the inefficiency of cell transformation and the difficulty in further mutating the library. Indeed, the larger size of ribosome display libraries (as many as 10^{15} different peptides) has led to the routine selection of high-affinity protein and peptide binders (K_ds in the pM and nM ranges [7–10]). Another proposed advantage [3] is compatibility with technology for single unnatural amino acid (AA)[1] incorporation by suppression of a stop codon [11–13], recently demonstrated [14,15] using biotinyl-lysine-tRNA

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[1] *Abbreviations used:* AA, amino acid; bM, biotin-labeled-methionine; DTT, dithiothreitol; fM, formylmethionine; MetRS, methionyl-tRNA synthetase; NHS, *N*-hydroxysuccinimide; RS, aminoacyl-tRNA synthetase; TCA, trichloroacetic acid.

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suppressor with a relative of ribosome display, termed "in vitro virus" or "mRNA display" [16–20].

A long-term goal [21] is to develop vast amplifiable libraries consisting mostly, if not entirely, of unnatural AAs to permit the directed evolution of peptidomimetic drug candidates (e.g., the orally available cyclosporin A [22]) that are less succeptible to proteases than natural peptides. Attempting this goal by further extension of existing display systems using suppression with unnatural AAs is problematic [23,24]. Nonsense suppression is limited to the three nonsense codons [25], and clever sense suppression strategies [15,26–32] suffer from competition with natural aminoacyl-tRNAs and aminoacyl-tRNA synthetases (RSs). For example, combining an extract partially depleted in natural tRNAs with an optimal sense codon–unnatural tRNA pair [15] resulted in biased, but not exclusive, unnatural AA incorporation at this codon [33]. Furthermore, engineering of new anticodons must circumvent inadvertent recognition by the RSs because the anticodon is an important recognition element for 17 of the 20 RSs [34]. A different approach to address the exclusivity and generalizability problems and make possible the genetically programmable syntheses of peptide analogs was therefore necessary [35]: a bottom-up approach avoiding all competing reactions using a simplified, reconstituted, RS-free, pure translation system [21]. Initial studies with this approach demonstrated the feasibility of making peptide analogs up to seven AAs long using natural and chemoenzymatically synthesized aminoacyl-tRNA substrates [35]. However, before peptide analog evolution can be achieved by this approach, it is first necessary to develop a new relative of ribosome display that uses a pure translation system for polypeptide display, termed "pure translation display" [21]. This in turn entails the solving of some practical limitations of existing purified translation systems lacking RSs (see Results and discussion).

Materials and methods

mRNAs

Genes encoding a 3:2 ratio of valines to threonines, termed poly(Val,Thr), were created from synthetic oligodeoxyribonucleotides by overlap extension PCR [36], nested PCR, and cloning in pUC19 plasmid. Five different codons were used for the two AAs (Fig. 1A) to minimize the repetitive nature of the sequence, yet it still proved difficult to obtain long inserts or to obtain genes without nonsense mutations. mRNAs were prepared by transcription of *Eco*RI-digested plasmids that

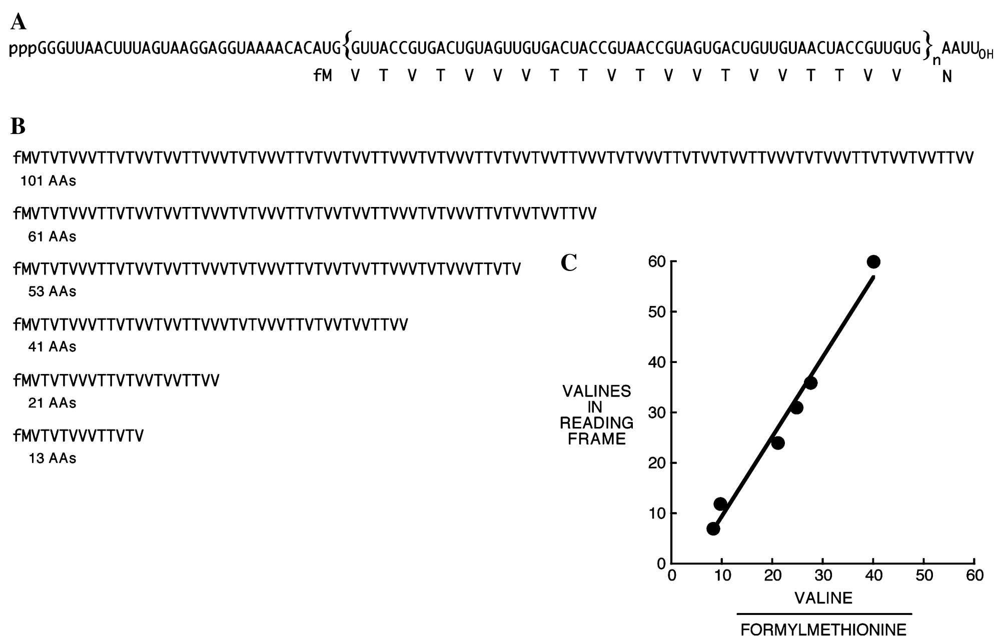

Fig. 1. Synthesis of long polypeptides with high processivity by a simple, fMet-initiated, RS-free, pure translation system. (A) Design of a family of mRNA sequences that differ by the number (n) of 60-nucleotide direct repeats (bracketed). (B) Predicted full-length translation products of obtained mRNAs when $tRNA^{Asn}$ is omitted (its codon is at the 3′ end of the mRNAs in (A), marked N). Note that the 53-mer and 13-mer reading frames were obtained fortuitously from mRNAs almost identical to that encoding the 61-mer due to inadvertant nonsense mutations (both giving UGA), while the 81-mer reading frame from a tetrameric direct repeat or reading frames longer than 101 AAs were not obtained. (C) Analysis of total translation products from each mRNA with the reading frames shown in (B) by TCA precipitation and a dual-label counting program that differentiates [^{3}H]valine from [^{35}S]formylmethione. Molar ratios of valine to formylmethione were calculated using the specific activities supplied by the manufacturer of the radioactive AAs (New England Nuclear). The plotted line-of-best-fit approximates the expected straight line of slope 1 calculated for 100% processivity.

bore unambiguous insert sequences using T7 RNA polymerase and unlabeled or [α-^{32}P]-labeled NTPs, and transcripts were purified [37].

BiotinylMet-tRNA$_i^{fMet}$

Met-tRNA$_i^{fMet}$ was prepared by charging pure tRNA$_i^{fMet}$ from *Escherichia coli* (Subriden RNA) with pure methionine (Sigma) using MetRS purified as described [38]. Met-tRNA$_i^{fMet}$ (19 μM) was labeled with sulfo-NHS-LC-biotin (1.1 mg/ml; Pierce) in $NaHCO_3$ (50 mM) at 0 °C for 40 min or less (minimizing hydrolysis of the aminoacyl linkage). The pH was then adjusted with excess NaOAc, pH 5, and product was separated from excess sulfo-NHS-LC-biotin by centrifugation through a Microcon 10 ultrafiltration device (10-kDa cutoff; Amicon). Labeling occurred at about 20% efficiency on the AA and did not occur on the tRNA body, as measured by acid urea PAGE [39]. Since only purified materials were used, the reaction product must have a blocked α-amino group (not an AA side-chain modification) that prevents incorporation of biotin anywhere in a translation product except for the N terminus.

Translations

Translations used components purified from *E. coli* [21]. The translations were performed for 30 min as described [35] without preincubation so that initiation and recycling should be rate-limiting [21], thereby preventing exhaustion of elongator substrates. Such exhaustion would lead to premature terminations and artifactually low processivities. Aminoacyl-tRNA concentrations were ~0.2 μM fMet-tRNA$_i^{fMet}$ (or biotinylMet-tRNA$_i^{fMet}$), ~1 μM Thr-tRNA$_3^{Thr}$ (or Thr-tRNA$_1^{Thr}$ (Subriden RNA) which recognizes the same codons), and ~1.5 μM Val-tRNA$_1^{Val}$. Products were analyzed by binding to TetraLink avidin beads (Promega) [21] or by trichloroacetic acid (TCA) precipitation on nitrocellulose filters. Because of the hydrophobicity of the longer products, analysis by various gel systems proved impractical. Some 20% of the elongator substrates were incorporated, assuming quantitative precipitation and recovery for analysis, and higher incorporations were obtained by substitution of the ribosomes with a more active preparation [40,41] kindly supplied by Dr. Martin Olsson and Dr. Måns Ehrenberg (results not shown).

Pure translation displays

The washing and elution steps built upon those used for ribosome display [42]. The immobilized target protein, avidin, in the form of TetraLink avidin beads (Promega), was first equilibrated with wash buffer (WB; 50 mM $Mg(OAc)_2$, 150 mM NaCl, 50 mM Hepes-KOH, pH 7.0, 10 mM DTT, 0.1% Tween 20). As a precautionary measure, the beads were then blocked at 4 °C for ~5 min by addition of a similar volume of a translation that was identical in composition to the test translations, except that the initiator was fMet-tRNA$_i^{fMet}$ and the mRNAs were nonradioactive. The blocked bead suspension was then divided into 10-μl aliquots. For biotin-blocked controls, translation-blocked bead aliquots were further blocked with a quarter volume of biotin (5 mM) at 37 °C for 5 min.

Libraries of mRNA/ribosome/peptidyl-tRNA complexes were prepared by translating in 5 μl a mixture of [^{32}P]-labeled mRNAs (e.g., Fig. 3B, "input mRNAs" lane) using biotinylMet-tRNA$_i^{fMet}$ for 30 min at 37 °C. The complexes were stabilized by chilling at 0 °C for 2 min, adjusting the Mg^{2+} concentration to 48 mM by addition of 95 μl ice-cold WB (using precooled pipette tips), and performing all remaining steps in a 4 °C room. The tubes were incubated for 5 min on ice and the solutions were then transferred (to leave behind any plastic-binding complexes) into the tubes containing the blocked beads and mixed by tapping every 10 min over a 40-min period at 4 °C. The beads were spun down and the supernatants were removed (e.g., Fig. 3B, "first wash" lane) and the beads were washed three times with 200 μl WB. mRNAs were eluted from bound complexes by addition of 40 μl elution buffer (EB; 100 mM EDTA, 150 mM NaCl, 50 mM Hepes-KOH, pH 7.0, 10 mM DTT, 0.1% Tween 20) and incubation for 5 min, the supernatants were recovered, and any residual contaminating beads were removed by filtration. The efficiency of recovery of the 101-AA-encoding mRNA was estimated by gel electrophoresis and autoradiography to be ~0.2%, similar to that of crude translation displays (the calculation can be made directly by inspection of the intensities of the bands in Fig. 3).

Results and discussion

Before attempting pure translation display in the absence of RSs, it was first necessary to extend the synthetic capabilities of RS-free translation systems. The existing system for programmable synthesis of peptide analogs was tested only for up to seven successive AA incorporations [35]. These short peptide analogs would be masked from display by the ribosome tunnel (~100 Å long ~30 extended AAs in the structure of the *Haloarcula marismortui* 50S subunit [43]). Experimentally, the number of spacer residues required to span the *E. coli* tunnel depends on the sequence, with reported lengths ranging from about 44 to 72 AAs [44]. Though products this long have been synthesized in purified systems containing all 20 RSs [38,45], processive synthesis of products this long by simplified fMet-initiated, pure translation systems without RS-catalyzed substrate recycling has been elu-

sive. The original simplified system synthesized tripeptides [46], but had processivities that were reportedly low for tetrapeptide syntheses [47] and indeterminate for longer polypeptides [48]. Addition of enigmatic factors termed EF-P, W, and Rescue was reportedly necessary to aid processivity [49,50]. Alternatively, a system that contained three RSs was limited by RNase degradation of mRNA [51]. Thus, we first needed to test the feasibility of long polypeptide synthesis in our RS-free, pure translation system.

In designing a spacer sequence to span the ribosome tunnel in a pure translation system, we avoided problematic mRNA and polypeptide sequences [52,53] and limited the number of AA-tRNA substrates necessary for its translation to two for simplicity. We took advantage of initial results with Val-$tRNA_1^{Val}$ and Thr-$tRNA_3^{Thr}$ substrates [21] and the predicted ease of analysis of their hydrophobic polymeric products by precipitation with TCA (by analogy with classic poly(Phe) assays [54]). mRNAs encoding repetitive spacers of poly(Val,Thr) were designed (Fig. 1A), and a series of constructs encoding polypeptides ranging from 13 to 101 residues in length were obtained (Fig. 1B). These spacer sequence mRNAs were then translated individually by our pure RS-free system under conditions where initiation and recycling should be rate-limiting (see Materials and methods). These translations yielded total products containing ratios of elongator [^{3}H]valine to initiator [^{35}S]formylmethione that were directly proportional to length, demonstrating high processivity (Fig. 1C).

Inspired by early display experiments that initiated ribosomal polypeptide synthesis with an antigenic unnatural AA carried by initiator tRNA [52,55], we chose to prepare an aminoacylated initiator tRNA capable of delivering a readily selectable biotinylated methionine residue (Fig. 2A) to the N terminus of the translation product of any mRNA template containing a ribosome binding site. Despite the bulky N-substitution, biotinylMet-$tRNA_i^{fMet}$ initiates ribosomal synthesis of

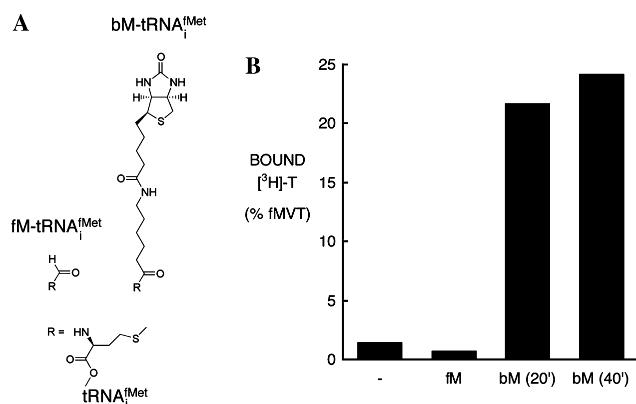

Fig. 2. Generalizable method for the cotranslational incorporation of biotin specifically at the N terminus of the translation product of any mRNA containing a suitable ribosome binding site. (A) As a substitute for formylMet-$tRNA_i^{fMet}$ (left) in translation initiation, biotinylMet-$tRNA_i^{fMet}$ (right) was prepared by labeling Met-$tRNA_i^{fMet}$ with sulfo-NHS-LC-biotin. (B) The products of 20- and 40-min labelings were tested for ribosomal incorporation into bMVT tripeptide programmed by mRNA mMVT [21] using an avidin-binding assay [21]. Binding in comparison with no initiator (-) or with wild-type formylmethionine initiator (fM) is shown. Calculations were performed by subtracting the background dpm of a control translation lacking mRNA (not shown) from the total dpm, and the resulting dpm were then compared as a percentage with the fMVT yield from a control translation assayed by cation-exchange chromatography [70] (not shown).

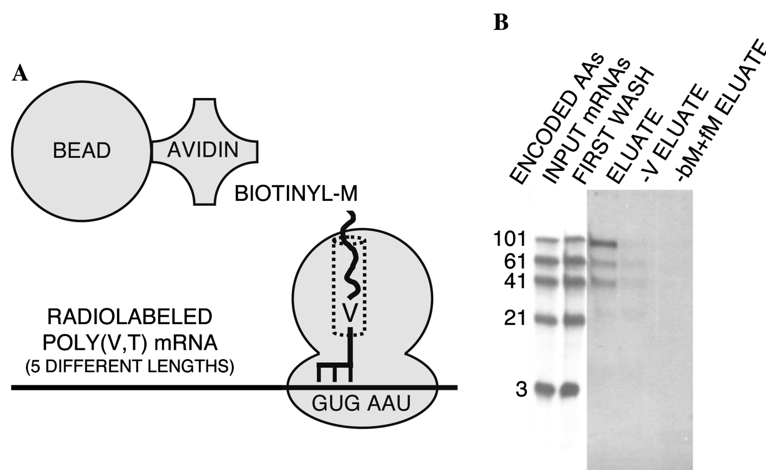

Fig. 3. Selection of mRNAs from a model mRNA library by pure translation display. (A) Schematic of the experiment that selects for ligand–mRNA complexes. In contrast to ribosome display, where mRNAs lacking stop codons in crude translation systems yield complexes with ribosomes paused at the 3′ ends of the mRNAs, pausing in pure translation display occurs within the mRNAs due to omission of cognate aminoacyl-tRNA(s). Though not illustrated here, the mRNA may be translated by multiple ribosomes to yield a polysome. (B). Analysis of stability of [^{32}P]-labeled mRNAs in the translation mixture and their selection by pure translation display by autoradiography of a representative urea polyacrylamide gel. The five input mRNAs encoding the number of AAs shown are described in Figs. 1 and 2 (the mRNAs encoding 53 or 13 AAs were omitted because they are indistinguishable in length from the mRNA encoding 61 AAs). The two lanes on the left had less exposure time (1/5th) and loading (1/100th). See text for details.

avidin-binding peptides (Fig. 2B) and is competent for initiation of translation with biotin when a model mRNA library is used (see below). BiotinylMet-tRNA$_i^{fMet}$ may also prove useful for proteomic studies, where elongation could be performed in a pure [38,45] or crude translation system to yield proteins for N-terminal immobilization or purification [56].

The above methods were then combined to determine whether polypeptide display could be performed in a pure translation system (Fig. 3A). Such an experiment tests whether components of crude translation systems absent from our pure system might be required to stabilize mRNA/ribosome/peptidyl-tRNA complexes (e.g., chaperones and targeting factors known to bind to the exit of the ribosome tunnel [53]) and measures the length of spacer sequence required for display. A mixture of radiolabeled poly(Val,Thr) mRNAs of various lengths (Fig. 3B, "input mRNAs" lane) was translated with biotinylMet-tRNA$_i^{fMet}$, Thr-tRNA$_1^{Thr}$, and Val-tRNA$_1^{Val}$, and members of this model mRNA library that encoded products long enough to span the ribosome tunnel for display were selected with avidin beads (Fig. 3A). The beads were washed and the captured mRNAs were eluted with EDTA, which dissociates mRNA/ribosome/peptidyl-tRNA complexes. The expectation was that the 101-mer and perhaps the 61-mer and the 41-mer AA-encoding mRNAs would be selected but not the shorter ones [44]. Thus, the shorter mRNAs are controls for the experiment and reveal the minimum spacer sequence necessary for display. Indeed, the selected mRNAs encoded 41 or more AAs (Fig. 3B, "eluate" lane), and the optimal spacer length was 101 AAs. Controls showed that RNase activity was undetectable in the 30-min translation (Fig. 3B, lane with "first wash" of beads), in marked contrast to crude systems [57]. Selection was abolished by preblocking of the avidin beads with biotin (not shown), by omitting Val-tRNA$_1^{Val}$ (Fig. 3B, "−V eluate" lane), and by substituting biotinylMet-tRNA$_i^{fMet}$ with fMet-tRNA$_i^{fMet}$ (Fig. 3B, "−bM+fM eluate" lane). Note that there are some very faint mRNA bands detectable in the controls (best seen in the "−V eluate" lane) that have a pattern like the "input mRNAs" lane, not like the "eluate" lane, implying that they are due to nonspecific background mRNA binding and elution. Just a single round of selection led to an enhancement of about 20-fold for mRNAs encoding 101 AAs compared to those encoding 21 or 3 AAs (Fig. 3B). The selectivity and recovery observed for our mRNAs encoding the 101-mer are comparable with the selectivities and efficiencies reported for crude translation displays [3,17,57].

Alternative formats for pure translation display are possible. For example, cyclic sequences could be displayed by incorporation of pairs of cysteines for disulfide bond formation [6]. Given that an RS-free pure translation system can support a display requiring synthesis of a long spacer, compatibility is anticipated with displays that circumvent the spacer requirement by covalent linkage of the polypeptide to the mRNA via DNA-puromycin [16,17,19] or derivatized AA-tRNA linkers [58] and subsequent removal of the ribosomes. Though these methods have the disadvantages of special chemistries and additional steps, they form more stable nucleic acid–polypeptide complexes for the selection step.

The potential advantages of pure versus crude translation displays derive from the greater control afforded over the translation step. Pure translation display should be more amenable to optimization because each component can be systematically varied and because competition can be avoided from endogenous termination factors (including tmRNA [57]), mRNAs, RNases, and proteases, thereby allowing efficient conversion of mRNA into stable mRNA/ribosome/peptidyl-tRNA complex [41]. It also should be more versatile because, in the absence of interfering RSs, mRNAs can be translated into products containing multiple unnatural AAs with high fidelity [35]. Though scalability was established in the existing system for programmable synthesis of peptide analogs, only 6 of the 64 codons were reassigned to unnatural AAs using chemosynthetic aminoacyl tRNAs [35]. Reassignment of many more codons is necessary for construction of diverse peptide analog libraries and the reiterative evolution of the codons of the libraries in an unrestricted manner. This could be achieved by construction of as few as 16 chemosynthetic aminoacyl-tRNAs containing the 16 different GNN anticodons so that each translates a different NNC/U codon pair. Reiterative mutations could be confined to essentially these same 32 codons using transition-prone PCR [59]. Selections may also be performed using natural elongator aminoacyl-tRNAs that have been chemically modified at their AA moieties [21,60]. Incorporation of multiple AA backbone analogs such as *N*-methyl amino acids [25,33,60,61] could increase protease resistance and membrane permeability [33,62–65], thereby allowing the directed evolution of peptidomimetic drug candidates [24]. Other analogs might aid the directed evolution of new catalysts [66].

Applications of pure translation display that are very different from peptide analog evolution are also foreseeable. For example, pure translation systems including all 20 RSs have been previously shown to synthesize proteins efficiently [38,45], satisfying a prerequisite for applications in proteomics [67–69].

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