

Puromycin technology for *in vitro* evolution and proteome exploration

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Abstract

Puromycin, an analogue of the 3' end of aminoacyl-tRNA, is transferred non-specifically to growing polypeptide chains, causing premature termination of translation. However, we found that, at very low concentration, puromycin is transferred specifically to the carboxyl (C-) terminus of the full-length protein. The term puromycin technology refers to methods which have been developed based on this property, including the *in vitro* virus (IVV) display technique, in which an mRNA (genotype) and its protein (phenotype) are linked via puromycin, and the technique of specific *in vitro* C-terminal protein labeling. Novel and convenient applications based on puromycin technology have been employed not only for evolutionary protein engineering, such as protein-directed selection, but also in various fields of proteomics research, such as fluorescence labeling, affinity purification (pull-down assay), protein-protein interaction analysis, and protein chips. Here, we review the properties of puromycin that make this technology possible, and the already extensive range of applications of this technology.

Keywords: puromycine, evolution, proteome, *in vitro* virus (IVV), C-terminal protein labeling

Introduction

Puromycin is a very powerful inhibitor of protein synthesis in both *in vivo* and *in vitro* translation systems [1-8]. Structurally, it resembles the 3' end of an aminoacyl-tRNA molecule [2], and it is able to enter the ribosomal A site to be transferred to nascent polypeptide chains by peptidyl transferase [9-11]. However, we showed that, at very low concentration in a cell-free translation system, puromycin is transferred specifically to the C-terminal of the full-length protein [12]. We utilized this finding to link mRNA and protein to form a so-called *in vitro* virus (IVV) for evolutionary protein engineering [13]. The IVV provides a system for mRNA display, in which a genotype molecule (mRNA) is linked to the corresponding phenotype molecule (protein) through puromycin in a cell-free translation system (Figure 1A) [13-14]. We also utilized the same property of puromycin

to develop a highly specific labeling system for proteins, in which puromycin derivatives bearing a fluorescein moiety are used to label the C-terminal end of a full-length protein (Figure 1B) [14-15].

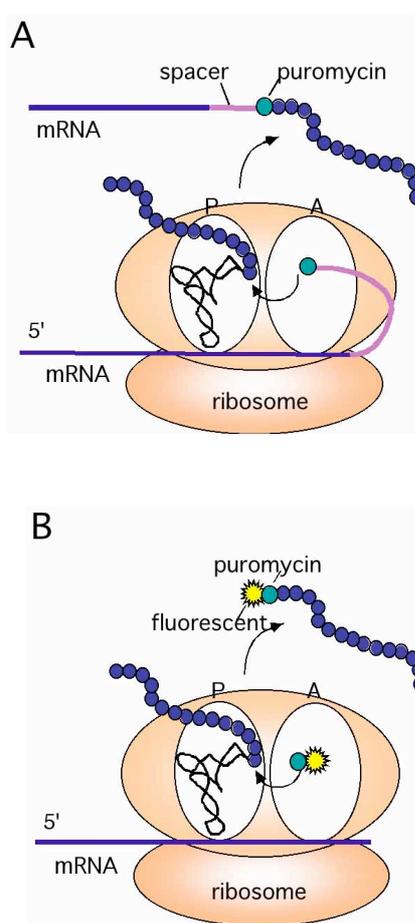


Figure 1. Puromycin technology; the C-terminal protein labeling and IVV methods. (A) IVV formation on the ribosome (1). Puromycin at the 3'-terminal end of a spacer (Fluor-PEG Puro; 14) ligated to mRNA can enter the ribosomal A-site to bind covalently to the C-terminal end of the encoded full-length protein in the ribosomal P-site. Fluor stands for fluorescein. Puro represents puromycin. (B) C-Terminal labeling of proteins on the ribosome (14,15). A puromycin derivative (Fluor-dCpuro; 16) can enter the ribosomal A-site to bind covalently to the C-terminal end of the protein in the ribosomal P-site.

The development of proteomics has led to an increasing interest in cell-free translation systems, because of their rapidity and ease of handling. Several new *in vitro* protein production technologies yielding large amounts of protein have been developed [16-17], and in combination with puromycin technology, have been utilized for evolutionary protein engineering [13-14, 18], as well as in proteomics. Recently, *in vitro* protein selection using mRNA display methods such as IVV [19-21] and RNA-peptide fusions [22-23] have been employed for the analysis of protein-protein interactions using cDNA libraries. This display technology for protein selection and screening has already achieved spectacular results. Moreover, we have reported various other applications, including *in vitro* analyses of protein-protein interactions using C-terminally labeled proteins with microarrays and fluorescence cross-correlation spectroscopy (FCCS) [24-25]. Here we present an overview of the present status and prospects of puromycin technology.

1. Puromycin technology

The antibiotic puromycin [1], which is an analogue of the 3' end of Tyr-tRNA^{Tyr} [2], acts in both prokaryotes and eukaryotes [3-5] as an inhibitor of peptidyl transferase [6-7]. It has two modes of inhibitory action. The first is by acting as an acceptor substrate which attacks peptidyl-tRNA (donor substrate) in the P site to form a nascent peptide [6-9]. The second is by competing with aminoacyl-tRNA for binding to the A' site, which is the binding site of the 3' end of aminoacyl-tRNA within the peptidyl transferase active site [10-12]. It has been reported that the polypeptides released by puromycin are not full-length protein [6]. Similarly, it has been shown that growing peptide chains on ribosomes are transferred to the α -amino group of puromycin, which interrupts the normal reaction of peptide bond formation [7]. Therefore, these conventional studies suggested that puromycin is a non-specific inhibitor of protein synthesis as a result of competition with aminoacyl-tRNA. However, since most of the studies on puromycin were performed at relatively high concentrations, the behavior of puromycin at lower concentrations was still an open question at that time. It had been reported that full-length protein which fails to be released from ribosomes at the final stage of protein synthesis, requires treatment with puromycin or RFs to be released [26-27]. These results led us to hypothesize that puromycin at very low concentrations, which would not effectively compete with aminoacyl-tRNA [12], might act as a non-inhibitor and bond specifically to

full-length protein at the stop codon.

Indeed, we confirmed that puromycin and its derivatives bond only to full-length protein at very low concentrations (such as 0.04 μ M), where they are "non-inhibitors" of protein synthesis, by using ³²P-labeled rCpPuro (2'-ribocytidylyl-(3'→5')-puromycin) in an *E. coli* S30 extract cell-free translation system [12]. Our results provided the first evidence that specific bonding of puromycin to the full-length protein occurs at the stop codon during the process of termination of protein synthesis at very low concentration. In other words, puromycin at sufficiently low concentrations only has the opportunity to be bonded to proteins at a stop codon, where it does not need to compete with aminoacyl-tRNA. Since termination is a relatively slow step involving a translational pause in eukaryotes [28-29] and *E. coli* [30], it is possible that puromycin even at very low concentrations can bind to the A' site, and compete with RFs to release the full-length protein from ribosomes. Accordingly, under these conditions, puromycin can be incorporated specifically at the C-terminus of the full-length protein. This concept is the basis of puromycin technology [12,14]. The combination of this puromycin technology with *in vitro* translation has yielded novel methods, such as IVV and C-terminal labeling techniques, for protein selection and screening [19-21], fluorescence labeling [19-21], and affinity purification (pull-down assay), as well as protein chips for proteomics [24,25], and superior methods for evolutionary protein engineering.

2. *In vitro* virus; IVV

For Darwinian evolution, genotype assignment to the phenotype is essential. Evolutionary molecular engineering through *in vitro* RNA selection using ribozyme-type assignment molecules was proposed [31-33] and shown to be effective [34-35]. However, greater progress has been achieved with *in vitro* protein selection using fusion-type assignment molecules, in which genotype molecules are bound to phenotype molecules, such as ribosome display [36], mRNA display, including IVV [12-14,18], and DNA display [37-38], based on cell-free translation systems. Since these display technologies are *in vitro* versions of phage display [39], permitting global search of a large sequence space, such as the whole protein sequence space.

We developed IVV, the first application of puromycin technology, for mRNA display in 1997 [13]. Shortly thereafter, RNA-peptide fusions were independently reported for mRNA display [18]. The principle of puromycin

technology has been applied in both methods, which are based on the use of puromycin as a physical linkage between mRNA and its encoded full-length protein to obtain assignment molecules. These methods have already achieved substantial results, though early work uncovered some problems in obtaining large libraries and highly efficient enrichment, as well as involving rather tedious processes [13,18]. The efficiency of the ligation between the mRNA and the puromycin spacer was initially very low (about 20-30%), but this was improved (to about 70%) by alternative ligation methods such as splint ligation [40] and photo-linked ligation [41]. Further, we have reported a highly efficient single-strand ligation (over 90%) using a fluorescein-conjugated polyethylene glycol puromycin spacer (PEG-Puro spacer; Figure 2A) [14]. The efficiency of fusion formation was initially less than 10% [13] or 1% [18], but this has been improved to 40% [39, 42-43]. In our experiments, we have obtained more than 70% fusion formation [14] through single-strand ligation using a flexible PEG-Puro spacer and an optimized mRNA template in a cell-free wheat germ translation system or rabbit reticulocyte lysate translation system.

We also demonstrated that single-strand ligation using the flexible fluorescein-conjugated

PEG-Puro spacer (Figure 2B) instead of DNA [13,18] or triethylene glycol (TEG) [39] spacer provides an improvement of mRNA stability and IVV formation [14]. Moreover, we found that mRNA having a 5'-untranslated region including SP6 promoter and Ω 29 enhancer (a part of tobacco mosaic virus Ω), and an A8 sequence (eight consecutive adenylate residues) without a stop codon at the 3' end is suitable for fusion formation [14]. The use of mRNA without a stop codon is preferable to establish fusions of mRNA and its encoded peptide or protein, because this avoids competition of puromycin with RFs [12,13]. Optimization of the 5'- and 3'-terminal sequences of mRNA templates offers the advantage of easy handling because of high efficiency of IVV formation, simplifying formerly tedious processes.

3. *In vitro* evolution and proteome exploration

In vitro display technology has many advantages [44]. It offers a greater library diversity (10^{12-13} /ml) compared with phage display [39]. In fact, various aptamers have been successfully evolved from random libraries using this approach [45-48]. Evolution of antibody mimics by using mRNA display [49] or ribosome display [50] and identification of epitope-like consensus motifs [51] have also been reported.

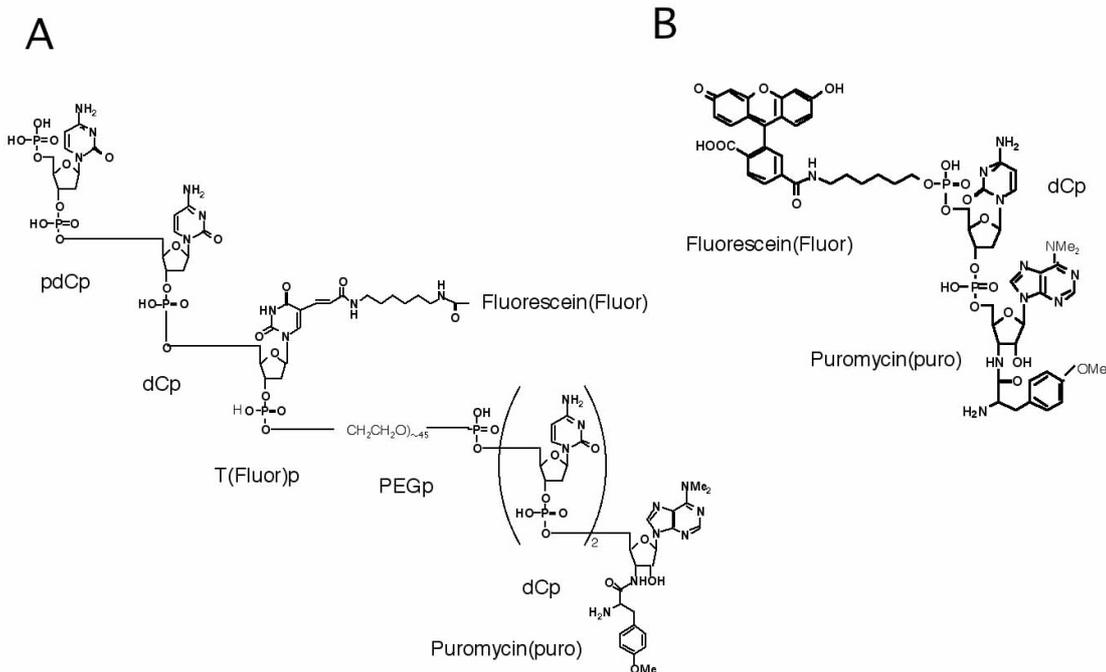


Figure 2. PEG puromycin spacer for the IVV method and puromycin analogues for the C-terminal protein labeling method. (A) The structure of PEG puromycin spacer. Fluor-PEG Puro spacer [p(dCp)2-T(Fluor)p-PEGp-(dCp)2-puromycin] was synthesized from Puro(Fmoc)-CPG, Polyethylene glycol (PEG, average mol. wt 2,000), deoxycytidine phosphoramidite (dC-amidite), thymidine(flourescein) phosphoramidite [T(Fluor)-amidite]. (B) The structure of fluorescence-labeled puromycin. A fluorophore (herein, fluorescein) was chemically joined to puromycin through a dC linker.

Screening for binding streptavidin aptamer provided 10,000-fold stronger binding than that obtained with the *in vivo* method [52], supporting the superiority of the method. Screening for ATP binding also gave good results [53]. In many cases, desired functional peptides or proteins have been obtained by the application of display technology.

mRNA display methods, including IVV, have been successfully used to analyze protein-protein interactions (PPI) [19-22], and drug-protein interactions [54], starting from a cDNA library. In order to adapt the technology to protein function research, such as PPI analysis, *in vivo* preparation of bait proteins used to be required before *in vitro*-based manipulation [22-23]. Our developed cell-free cotranslation technique with IVV (Figure 3) has provided a totally *in vitro* manipulation that is suitable for high-throughput, genome-wide analysis with multiple bait proteins as a result of *in vitro* bait translation instead of *in vivo* bait preparation [19]. Cotranslation of bait and prey proteins should also be advantageous for the formation of protein complexes [55-56]. This approach currently appears to offer the best opportunity to obtain a comprehensive data set including not only direct, but also indirect interactions in a single experiment.

The display technology can also be applied to protein chip systems. A microarray in which mRNA-peptide fusions are immobilized by hybridization to a surface-bound DNA capture probe has been developed, resulting in an addressable mRNA-peptide fusion microarray [55]. This provides a protein chip in which the protein is linked to its genetic information. This system can be used to analyze genetic information associated with interactions of a large-diversity protein library versus a large-diversity library of nucleotides, sugars, etc. This is potentially a high-throughput method, which should contribute greatly to proteomics. Epitope-mRNA fusions have been bound to the surface and the interaction with fluorescence-labeled antibody has been analyzed [57].

4. Full-length protein labeling at the C-terminal end

As already mentioned, puromycin and its derivatives at low concentrations can bind specifically to the carboxyl terminus of full-length proteins [12] and a fluorescein-puromycin conjugate has been successfully used for the C-terminal fluorescein labeling of proteins [15]. This fluorescence-labeling method is useful for analyzing protein-protein or protein-DNA interactions with DNA microarrays or with

fluorescence cross-correlation spectroscopy (FCCS), and for pull-down assay [24].

The efficiency of C-terminal labeling is reported to be between 50% and 95% [15]. To confirm the general utility of this labeling method, we chemically synthesized various puromycin analogs in which one or two (deoxy)cytidylic acid(s) (i.e., dC, dCdC, rC, or rCrC) were inserted between the fluorophore (fluorescein, RhG, TAMRA, Cy3, or Cy5) and puromycin as a linker to enhance the incorporation of puromycin analogs into proteins by mimicking the CCA sequence at the 3' end of tRNA [24]. These puromycin analogs were added to the wheat germ *in vitro* translation system [17]. Among them, we found that the Fluor-dC-puromycin conjugates (Figure 2B) gave the best yield because of the use of the dC linker. The dC linker should be inferior as an inhibitor to the rC linker in the middle of protein synthesis and superior to the rC linker as a non-inhibitor for specific bonding to the full-length protein in the final stage of protein synthesis. In addition, we have optimized an mRNA template having a certain sequence at the 3' end (XA8 sequence; XhoI and eight consecutive adenylate residues) to provide 3-

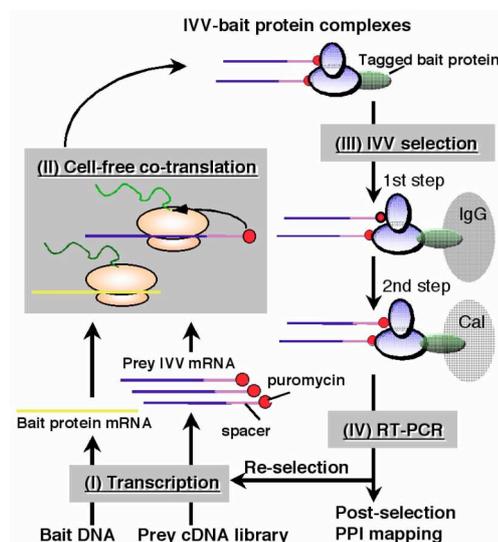


Figure 3. IVV selection based on cell-free cotranslation. The selection procedure is composed of (I) transcription, (II) cell-free cotranslation, coupling translation and interaction of tagged bait protein and prey IVV library to form complexes, (III) IVV two-step selection (Rigaut et al. 1999), and (IV) RT-PCR to amplify the library. IgG beads (IgG) for the protein A (zz domain) tag are used for the 1st step of the purification, and calmodulin beads (Cal) for the calmodulin binding protein (CBP) tag are used for the 2nd step of the purification.

5-fold higher formation efficiency of C-terminally labeled proteins [14]. We concluded that mRNA templates with the XA8 sequence as a 3' end and SP6 + Ω29 as a 5' UTR are clearly preferable for the C-terminal labeling of proteins to facilitate high-throughput proteome exploration.

5. Proteome exploration using c-terminal protein labeling

This C-terminus labeling method has many advantages. Because the protein is labeled only at the C-terminus, most proteins can maintain their functionality. The method is suitable for quantitative analysis because there is a single fluorophore on each protein molecule. The effect of labeling on the protein activity should be smaller than with the GFP fusion method, because of the much smaller size of the label. We have shown that C-terminal fluorescence labeling is very useful for many applications, including detection of protein-protein or protein-DNA interactions with a DNA microarray or with FCCS, and pull-down assay [24-25].

Recently, the C-terminal labeling method has been applied to observe protein expression *in vivo* [58-59]. The X-dC-puromycin conjugates bearing various fluorescent and biotin moieties (Figure 2B) have been shown to be a new class of reagents for studying protein expression not only *in vitro*, but also *in vivo* [58]. These compounds are readily incorporated into expressed protein products in cell lysates *in vitro* and efficiently cross the cell membrane to function in protein synthesis *in vivo*. This method was used to examine whether dopamine receptor activation stimulates local protein synthesis in living cells [59]. The system enables direct monitoring and imaging of protein expression with fluorescent puromycin-labeled protein, and has the potential to provide high spatial and temporal resolution in living cells.

Conclusions and outlook

Puromycin technology in combination with cell-free translation systems allows the processing of huge libraries, is not limited by cellular transformations, and is not biased by *in vivo* environments. *In vitro* display technologies using this approach for mRNA display, such as IVV, have proved to be valuable tools for many applications other than merely selecting polypeptide binders. They have great potential for directed evolution of protein stability and affinity, for the selection of enzymatic activities, antibodies, and functional peptides from random-peptide libraries, and for proteomics research, including protein-protein interactions, drug-protein interactions, and potentially

DNA-protein interactions from cDNA libraries. The C-terminal protein labeling technology is very useful for analyzing protein-protein or protein-DNA interactions with DNA microarrays or with FCCS in model systems, and is expected to have wide applicability. Thus, puromycin technology is considered to be a next-generation technology that will have a great impact in many areas of biotechnology, medicine and proteomics.

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