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Breakthroughs and Views

Next-generation protein-handling method: puromycin analogue technology☆

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Abstract

Puromycin is a well-known antibiotic that inhibits protein synthesis by competitive incorporation against an aminoacyl tRNA on the ribosome A site. Novel technology using this property of puromycin has been developed for convenient handling methods in protein research. Puromycin modified with another molecule is incorporated into a protein at the C-terminus, thus linking the desired molecule to the protein. Combination of in vitro translation with puromycin analogues has resulted in novel technologies such as display technology for screening, fluorescence labeling, affinity purification, and protein chip for proteomics.

Keywords: Fluorescence label; Affinity chromatography; mRNA display; In vitro translation; Protein chip; Proteomics

In the post genome era, the importance of protein research in biotechnology is increasing. Therefore, development of a next-generation protein-handling method is expected. Puromycin is a well-known antibiotic that inhibits protein synthesis by competitive incorporation against an aminoacyl tRNA on the ribosome A site [1] and at low concentrations is apt to bind specifically to the C-terminus of a full-length protein [2]. Novel technologies using these properties of puromycin have been developed in protein research. Puromycin modified with another molecule is incorporated into a protein at the C-terminus, linking the desired molecule to the protein. This is the principle of puromycin analogue technology. Protein production by an in vitro translation system becomes important because of its rapidity and ease of handling. Several new methods yielding large amounts of protein have been developed [3,4]. Novel technologies have emerged from combining the in vitro translation technique with the puromycin analogue technique. A display technology for screening has already achieved great results. Other useful applications include fluorescence labeling, affinity purification, and protein chips for proteomics. Here we report the present status and future prospects of puromycin analogue technology.

C-terminus fluorescence labeling

Normally, puromycin inhibits protein synthesis, and premature proteins are produced. Puromycin at low concentrations, however, is apt to bind specifically to the C-terminus of a full-length protein [2]. Translation of mRNA with puromycin–fluorescein conjugate (“Fluorpuro”) gives a fluorescein-labeled protein [5]. The labeling efficiency is reported to be between 50% and 95%[5]. Furthermore, the dissociation constant can be determined using the labeled protein and fluorescence polarization measurement [5]. This C-terminus labeling method has many advantages. Because the protein is labeled only at the C-terminus, most proteins maintain their functionality. The method is suitable for quantitative analysis because there is one fluorophore on a protein molecule. The affect of labeling on the protein activity may be smaller than with the GFP fusion method because of the much smaller size of the label.

☆ *Abbreviations:* ORF, open read frame; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GFP, green fluorescent protein; FITC, fluorescein isothiocyanate; SA, streptavidin.

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Because this label is very stable, it emits fluorescence under denaturing conditions, e.g., SDS–PAGE. Fluorescence labeling of a protein with the C-terminus in the core of the molecule is difficult, but many proteins have been tested without such difficulty.

Other disadvantages of this method are as follows. The excess molarity of the fluorophore–puromycin conjugate must be added to the protein, and some fluorophores bind to the protein nonspecifically in the in vitro translation mixture. Therefore, separation of the fluorophore–puromycin conjugate from the labeled protein is difficult. In many applications, it is not a matter of importance, but in some cases, a residual of the free fluorophore–puromycin conjugate may disturb the analysis. We developed a method that avoids this drawback. Once an RNA–protein fusion is formed using a fluorophore linker (DNA oligomer, no need for excess fluorophore), we digest the linker DNA. Thus, the fluorescence-labeled protein is disturbed less by the residual fluorophore [6].

The most successful use of C-terminus fluorescence labeling was as an application in single-molecule imaging. Analysis of protein–protein interactions is important for understanding a genetic network and the C-terminus fluorescence labeling is suitable for such a purpose. By combining this technology with single-molecule imaging, the physicochemical properties of a single molecule can be observed conveniently. Movement of kinesin along a microtubule has been observed at the single-molecule level [7]. The experiment was straightforward preparation of a labeled protein by in vitro translation of mRNA with Cy5-dC-puromycin (Fig. 1), gel filtration, and single-molecule imaging. These processes took a total of only 2 h [7].

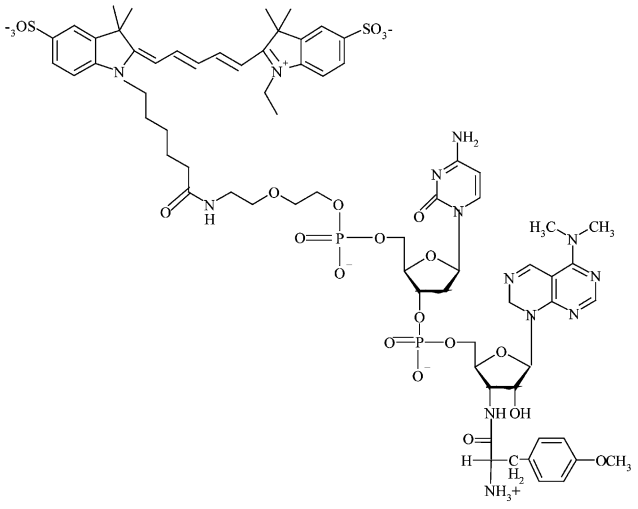

Fig. 1. The structure of Cy5-dC-puromycin, which is a typical fluorescent reagent in puromycin technology. On a ribosome, this molecule is incorporated into a protein at the C-terminus. Thus, the protein is fluorescently labeled.

Various fluorophore (fluorescein, rhodamine green, TAMRA, Cy3, and Cy5)–puromycin conjugates have been examined and function well [8]. Various linkers (dC, dCdC, rC, rCrC) between puromycin and fluorophore have also been tested to increase labeling efficiency. Among these dC linker gave the best result. The reason may be its accessibility to the ribosome A site.

The usefulness of this fluorescence-labeling method for analyzing protein–protein or protein–DNA interactions with a DNA micro array or with fluorescence cross-correlation spectroscopy in model systems has been reported [8]. Although only model system demonstrations have been presented, they showed, that C-terminus fluorescence labeling is very useful for many applications.

Rapid protein purification

The puromycin analogue technique is also suitable for use in the novel rapid protein purification method. Present affinity chromatographic methods have some weak points; for example, the His-tag has high background, and epitope tags require expensive antibodies. Our method has resolved these points. We synthesized puromycin-dC-biotin conjugate for the purpose of C-terminal affinity tagging. Synthesized C-terminus biotin-labeled proteins were purified with a soft link SA column (Promega) and desalted with a S-200 column (Amersham Pharmacia). Purified proteins had single bands on SDS–PAGE. These processes took only 2 h under very mild conditions [6]. If biotinylation is undesirable, biotin can be replaced with other tags such as poly(dA). We also synthesized puromycin–2′-*O*-methyl-C-FITC-dA18-biotin. This construct was easily devoid of biotin and poly(dA) by digestion with DNase, but it maintained FITC because 2′-*O*-methyl-C was not digested by DNase. Puromycin–2′-*O*-methyl-C was incorporated into a protein well [6], and sequential purification based on the poly(dA) moiety and the biotin moiety increased the purification level.

Display technology

Evolutionary molecular engineering has demonstrated its effectiveness in the design of novel functional biopolymers. The Darwinian selection process for proteins or peptides requires some strategy for linking between phenotype and genotype. Thus, some display technology is necessary. Display technology such as phage display [9] is an excellent method for screening a novel functional protein from a random library and has given good results.

An in vitro version of the phage display has been developed. Named in vitro virus [10] or RNA–peptide

fusion [11], this method has recently been called mRNA display. The primary construct is mRNA with puromycin in the 3′ end via a DNA spacer. In vitro translation of this primary construct gives an mRNA–protein covalent fusion. At the end of translation, a ribosome reaches theRNA/DNA junction and pauses, because DNA cannot be translated [11]. This pause allows the incorporation of the puromycin. Early reports described some problems, but they have now been resolved. The efficiency of the ligation between the mRNA and the puromycin linker was low; this efficiency was improved by the introduction of a novel ligation method [12,13]. The efficiency of fusion formation was less than 1% [11], but the efficiency has improved up to 40% [14–16]. In our experiments, the efficiency has improved even more—about 50% fusion formation using wheat germ extract or rabbit reticulocyte lysate. This improvement was realized by optimization of various points: spacer length, posttranslational incubation conditions, spacer flexibility, base sequence of the translational enhancer, etc.

This method has many advantages. It dramatically increased library diversity (about 10^6 increase in diversity from phage display [11]), system flexibility, and usable unnatural amino acid [17], and decreased screening cycle times. In fact, various aptamers have successfully evolved from a random library [18–21] or a cDNA library [22,23] in this approach. Evolution of antibody mimics [24] and identification of epitope-like consensus motifs [25] were also performed. Screening for binding streptavidin aptamer was performed, and the resulting product was 10,000-fold stronger than that obtained with the in vivo method [17]. This demonstrates the superiority of the method. Screening for ATP binding also gave a good result [18]. Normally, in a random library there are matched aptamers at a ratio of $1/10^{10}$ [18]. In most cases, the desired functional protein will be obtained with this method.

A superior system, cDNA–protein fusion [26] and in vitro DNA virus (Fig. 2) [27,28], has also been developed. These constructs tolerate more difficult selective conditions. Such methods have dramatically changed protein-screening techniques and will be the mainstream of protein screening technology. It is expected that routine work will give a novel functional protein.

Library construction

In an application of RNA–peptide fusion [11], a long ORF library must be created [29]. To screen and evolve long peptides of large diversity, a long ORF random oligonucleotide library is necessary. In standard chemical oligonucleotide synthesis, the library has stop codons at high frequency, making screening efficiency lower.

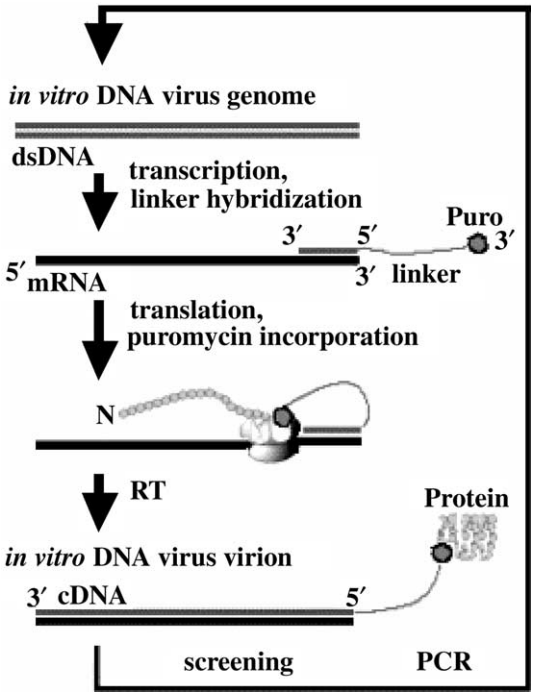

Fig. 2. "Life cycle" of "in vitro virus." This is an in vitro version of the phage display using puromycin technology. On a ribosome, puromycin linked to mRNA is incorporated into the nascent protein at the C-terminus. Thus, the protein is linked to the mRNA (or cDNA) via puromycin.

In this context, a preselected random library using the mRNA display was reported. Sequences containing any stop codons cannot make mRNA–peptide fusions. Thus, the selection of mRNA–peptide fusions removes any stop codons. This technique yields a long ORF random library of diversity greater than 10^{13}. Libraries of this type have given great results in in vitro evolution [18,19].

Protein chip

Puromycin analogue technology can also be applied to protein chip production. A microarray in which mRNA–peptide fusions are immobilized on the surface has been developed [30]. The mRNA–peptide is immobilized by hybridization to a surface-bound DNA capture probe, resulting in an addressable mRNA–peptide fusion microarray. This is a protein chip in which the protein is linked to its genetic information [30]. This system can be used to analyze genetic information in interactions of a large diversity protein library versus a large diversity library of nucleotides, sugars, etc. This is potentially a high-throughput method and will contribute greatly to proteomics. Epitope–mRNA fusions were bound to the surface and the interaction with fluorescence-labeled antibody was analyzed [30].

Approach to wetware artificial life

In vitro virus [10] can be regarded as the simplest virus, with a single gene and a single coat protein, and its "life cycle" is turned over in vitro, which means its "host" is a test tube. Moreover, because in vitro DNA virus [27] is free from the necessity of mRNA modifications—that is, a puromycin linker is attached to the mRNA only by hybridization—in vitro virus will be the next step toward autonomous evolution, such as in vivo viral evolution in a cellstat [31]. By combining the in vitro DNA virus with an isothermal amplification method for RNA/DNA such as 3SR (self-sustained sequence replication) [32], a continuous "culture" of the in vitro virus may be realized. This will be regarded as artificial life—a virus parasitic on a test tube. A flow reactor for this viral life cycle will be a "natural selection"-type in vitro evolution reactor for protein evolution.

Conclusion

A next-generation technology, the puromycin analogue method, dramatically extends protein-handling technologies in many fields.

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