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Biochemical and Biophysical Research Communications 337 (2005) 325–329
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Effect of energy source on the efficiency of translational termination during cell-free protein synthesis

Jin-Ho Ahn [a], Cha-Yong Choi [a,b], Dong-Myung Kim [c,*]

[a] *Interdisciplinary Program for Biochemical Engineering and Biotechnology, College of Engineering, Seoul National University, Seoul 151-742, Republic of Korea*
[b] *School of Chemical and Biological Engineering, College of Engineering, Seoul National University, Seoul 151-742, Republic of Korea*
[c] *Department of Fine Chemical Engineering and Chemistry, Chungnam National University, Daejeon 305-764, Republic of Korea*

Received 9 September 2005
Available online 19 September 2005

Abstract

We studied how the fidelity of translation termination is affected by the method of ATP regeneration during cell-free protein synthesis. During the in vivo expression of hEPO, whose termination is directed by the UGA codon, we found that substantial proportions of the translational products showed a larger molecular weight than expected. Similar results were obtained in a cell-free synthesis reaction using phosphoenol pyruvate (PEP) or 3-phosphoglycerate (3PG) for ATP regeneration. However, when the energy source was switched to creatine phosphate (CP), the readthrough of the UGA codon was completely repressed and only the target protein of the correct size was expressed in a high yield. To the best of our knowledge, this is the first report describing the relationship between the regeneration of nucleotide triphosphates and protein readthrough, and we also believe that the discovery would pave the way to the selective and efficient expression of target proteins in cell-free protein synthesis systems.

Keywords: Cell-free protein synthesis; Stop codon; Release factor; Energy source; ATP regeneration; Readthrough; Creatine phosphate; PEP; 3-Phosphoglycerate

Nowadays, cell-free protein synthesis is readily accepted as an alternative method of preparing protein molecules, especially in cases where the simultaneous expression of multiple proteins is demanded. This technique provides a versatile platform for the rapid translation of genetic information into protein molecules within a matter of hours. Moreover, the recent development of highly productive protocols for cell-free synthesis makes its practical application more feasible [1–4]. However, in contrast to the remarkable progress that has been made in improving the productivity of cell-free protein synthesis, little attention has been paid to the 'quality' of the cell-free synthesized protein.

The correct termination of translation is crucial for the homogeneous expression of a recombinant protein from a given DNA template. Translational termination is a complex process that requires the participation of several protein factors [5]. In normal wild-type *Escherichia coli*, there are no tRNAs that decode the three stop codons, viz. UAA, UAG, and UGA. Instead, the stop codons are decoded by release factor 1 (UAG, UAA) or 2 (UGA, UAA). The termination reaction at all three of these stop codons is stimulated by RF3 [6,7]. However, the completion of translation often fails with the result that larger products are produced, particularly when translational termination is directed by the UGA codon [8–11]. Since, in most of the currently available protocols, cell-free protein synthesis is mediated by the crude extract derived from the cytosol of *E. coli* (generally referred to as S30 extract), most of the problems encountered in in vivo expression are also likely to be observed during cell-free protein synthesis. Indeed,

* Corresponding author. Fax: +82 42 823 7692.
E-mail address: dmkim@cnu.ac.kr (D.-M. Kim).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.09.061

in our attempts to express plasmids coding for different proteins with the UGA triplet as an in-frame stop codon, products with an extended protein sequence were detected either in the cultivated cells or in the cell-free synthesis reaction.

In this study, we found that the issue of UGA readthrough can be effectively addressed in a cell-free protein synthesis system by employing an appropriate ATP regeneration method. For instance, while more than 20% of the cell-free synthesized protein exhibited a larger molecular weight when the ATP regeneration was supported by 33 mM PEP or 3PG, virtually all of the translation product was found to have the correct size in a reaction utilizing CP. The total amount of the synthesized protein was also significantly higher when CP was used as an energy source. Thus, by employing an optimized ATP regeneration method, we were able to maximize the productivity of the correctly terminated target protein.

Materials and methods

Materials. ATP, GTP, UTP, CTP, phosphoenol pyruvate (PEP), creatine phosphate (CP), and *E. coli* total tRNA mixture were purchased from Roche Applied Science. L-[U-^{14}C]Leucine (11.9 GBq/mmol) was obtained from Amersham Biosciences. All other reagents were purchased from Sigma. T7 RNA polymerase [12] and S30 extract [13] were prepared according to the previously reported protocols with minor modifications.

Construction of plasmids. The plasmids and primers used in this study are listed in Table 1. Cloning was performed by routine procedures [14]. All possible UGAN stop signals and the sequences of CAT and EGFP were amplified by PCR and inserted into the vector, pK7, between the *Nde*I and *Sal*I sites.

In vivo and cell-free synthesis of recombinant proteins. Escherichia coli cells [strain BL21(DE3)] were transformed with pK7EPO. While culturing them in LB medium, when OD_{600} reached 0.6, the expression of T7 RNA polymerase was induced with 1 mM IPTG for 2 h.

A standard reaction mixture was used for the cell-free protein synthesis and consisted of the following components in a total volume of 15 μl; 57 mM Hepes/KOH at pH 8.2, 1.2 mM ATP, 0.85 mM each of GTP, UTP, and CTP, 1.7 mM dithiothreitol, 80 mM ammonium acetate, 0.17 mg/ml *E. coli* total tRNA mixture (from strain MRE 600), 34 μg/ml L-5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid), 6.7 μg/ml plasmid, 33 μg/ml T7 RNA polymerase, 0.5 mM each of the unlabeled amino acids, 4 μl S30 extract, and the indicated amounts of the energy sources depending on the experiments.

Analysis of the expressed proteins. Proteins were run on a 13% Tricine–SDS–polyacrylamide gel [15], and Western blot analysis was performed with anti-EPO antibody or anti-His antibody. The total amount of cell-free synthesized protein was estimated by measuring the TCA-precipitable radioactivities as described in a previous report [13] and the residual ATP was estimated according to the previously reported protocols [16]. Image analysis was performed using ImageMaster 2D Platinum 5.0 (Amersham Biosciences).

Results

Readthrough of UGA codon frequently occurs either during in vivo or in vitro protein expression

The nucleotide sequence of human erythropoietin (hEPO) was cloned into the pK7 plasmid [17] between the *Nde*I and *Sal*I sites. The resulting construct (pK7hEPO) carries the ORF between the T7 promoter and the T7 terminator, and the termination of translation is directed by the UGA stop codon (Fig. 1), while an additional stop codon (UAA) is present in-frame 64 bps downstream of the UGA stop codon. Fig. 2A shows the result of the expression of hEPO in *E. coli* cells [BL21(DE3)]. Following induction with 1.0 mM IPTG, two types of translation products were detected on SDS–PAGE gel. In addition to the band with the expected molecular weight (18.5 kDa), an additional band whose molecular weight was approximately 2 kDa higher was also detected. The difference in molecular weight between these two bands correlates well with the distance between the first (UGA) and second (UAA) stop codons, indicating that the larger product is generated by the readthrough of the UGA codon. From the densitometric analysis of the Western blot results, approximately 20% of the expressed protein was found to be the readthrough product.

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Readthrough of the UGA codon was also observed when the same plasmid was expressed in the standard

Table 1
Plasmids and primers used in this study

Name	Description	Reference or source
Plasmids		
pK7EPO	EPO (0.501 kb) cloned into *Nde*I/*Sal*I sites of pK7, UGAA stop codon	[17]
pK7EPO-TGAT	UGAU stop codon	This study
pK7EPO-TGAC	UGAC stop codon	This study
pK7EPO-TGAG	UGAG stop codon	This study
pK7CAT-TGAA	CAT (0.657 kb) cloned into *Nde*I/*Sal*I sites of pK7, UGAA stop codon, N-terminal his-tag	This study
pK7EGFP-TGAA	EGFP (0.745 kb) cloned into *Nde*I/*Sal*I sites of pK7, UGAA stop codon, N-terminal his-tag	This study
Primers		
T7P-for	5′-TAATACGACTCACTATAGG-3′	
EPO-TGAT-rev	5′-CTTTGTTAGCAGCCGGTCGACATCATCTGT CCCCTGTCCT-3′	
EPO-TGAC-rev	5′-CTTTGTTAGCAGCCGGTCGACGTCATCTGT CCCCTGTCCT-3′	
EPO-TGAG-rev	5′-CTTTGTTAGCAGCCGGTCGACCTCATCTGTCCCCTGTCCT-3′	
CAT-TGAA-rev	5′-AAAAAAGTCGACTTCACGCCCCGCCCTGCCACTC-3′	
EGFP-TGAA	5′-AAAAAAGTCGACTTCACTTGTACAGCTCGTCCAT-3′	

T7 promoter

TCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTT

RBS Nde I

TCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATG

EPO *Opal stop*

GCC CCA CCA CGC CTC--------TGC AGG ACA GGG GAC AGA **TGA** **A**GT

CGA CCG GCT GCT AAC AAA GCC CGA AAG GAA GCT GAG TTG GCT

Ocher stop

GCT GCC ACC GCT GAG CAA **TAA** CTA GCA **TAA**

Fig. 1. Nucleotide sequence around the stop codon of pK7EPO. Stop codons are represented in bold letters.

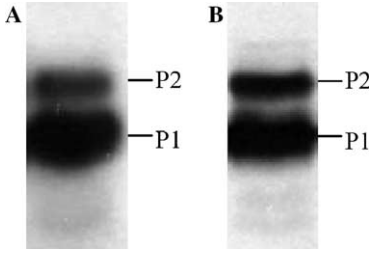

Fig. 2. Readthrough of UGA codon during in vivo and in vitro protein expression. (A) *Escherichia coli* cells [strain BL21(DE3)] were transformed with pK7EPO. While culturing them in LB medium, when OD_{600} reached 0.6, the expression of T7 RNA polymerase was induced with 1 mM IPTG. The expression level was analyzed by Tricine–SDS–PAGE/Western blot analysis. (B) The incubated reaction mixture from the cell-free synthesis (2 μl from 15 μl reactions) was evaluated using Tricine–SDS–PAGE/Western blot analysis. P1 and P2 represent the correctly terminated and readthrough hEPO, respectively.

cell-free synthesis reaction utilizing 33 mM PEP as an energy source. After 2 h of incubation, 90 μg/ml of the protein was synthesized, approximately 30% of which was a readthrough product (Fig. 2B). As was also observed in the case of its in vivo expression, the relative amount of the correctly terminated protein showed little dependency upon the reaction temperature (data not shown). However, during the investigations designed to optimize the reaction conditions so as to minimize the synthesis of the readthrough product, we found that the occurrence of readthrough is affected by the type of energy source that is used for the regeneration of the nucleotide triphosphates during the cell-free protein synthesis.

Initially, we compared the ability of three compounds containing high-energy phosphate bonds (PEP, CP, and 3PG) to support ATP regeneration and the synthesis of hEPO. At their optimal concentrations, it turned out that creatine phosphate (66 mM) had a higher productivity than the other compounds. 3PG (33 mM) was more efficient than PEP (33 mM), but not as efficient as 66 mM CP. A comparison of the time-course of the ATP level with these energy sources indicated that the observed relative productivities were closely related to the ATP level during the synthesis reactions (Fig. 3). Surprisingly, in the reaction utilizing creatine phosphate, not only was the overall productivity enhanced, but the generation of the readthrough product was also markedly repressed. In contrast to the results obtained when PEP or 3PG was utilized in the reactions, the translation product from the CP-utilizing reaction showed a single molecular weight corresponding to the expected size of hEPO.

In order to determine whether the readthrough of the UGA codon and its repression in the presence of creatine phosphate are limited to the expression of hEPO, different proteins were expressed in the presence of PEP, 3PG or CP and the translation products were analyzed by Western blot analysis. As shown in Fig. 4, readthrough products were found in the case of all of the reactions conducted with PEP or 3PG, and the frequency of readthrough showed little variation between the reactions. In contrast, readthrough products were not observed in all of the reactions that used CP as an energy source. These results imply that the readthrough of the UGA codon is completely repressed only when CP is used for NTP regeneration, and that this phenomenon is not limited to a specific protein.

There have been a few publications which reported that the decoding efficiency of stop signals is significantly influenced by the identity of the base following the stop codon. For instance, Poole et al. [18] showed that the termination efficiency of the maltose binding protein (MBP) varies depending on the nucleotide situated next to the UGA codon, and is in the order of U > G > A > C. Such a context effect was also observed in our cell-free synthesis system in the presence of PEP or 3PG acting as an energy source (Fig. 5). However, the dependence of the efficiency of termination on the nucleotides was different, indicating that

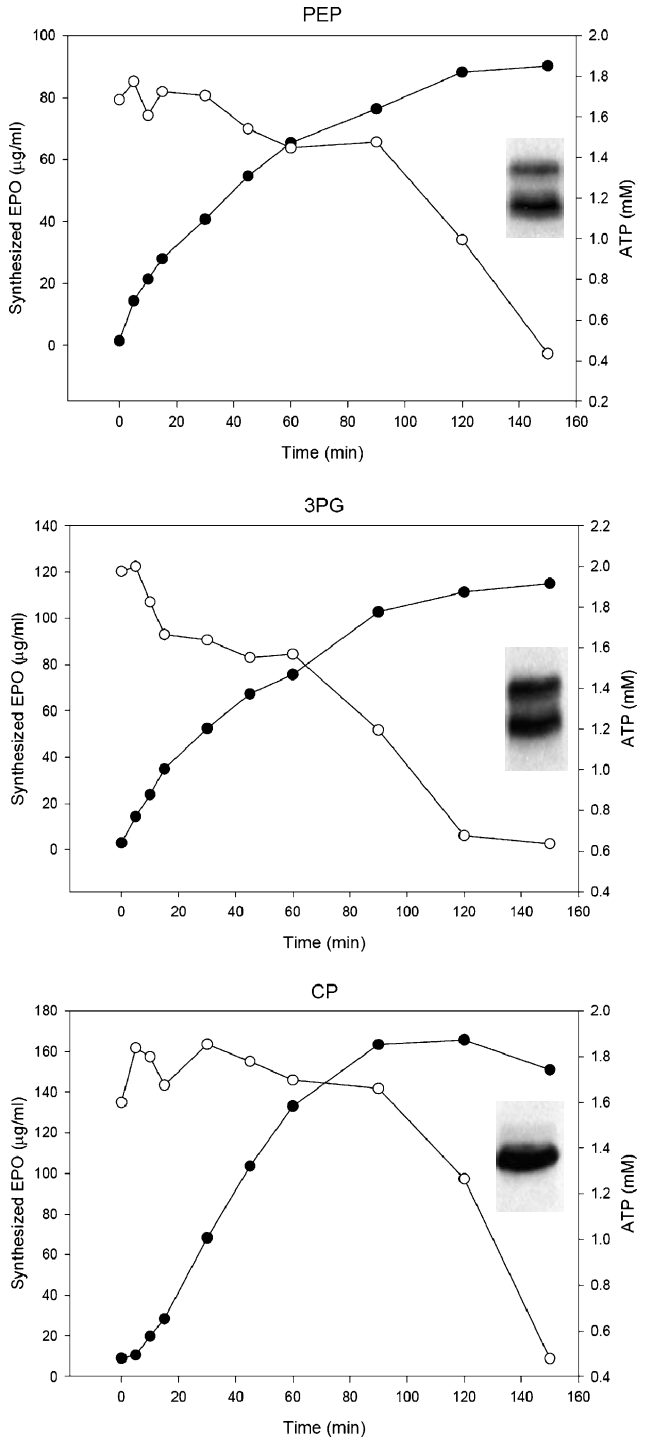

Fig. 3. Time-course analysis of protein synthesis, ATP level, and readthrough efficiency in a cell-free protein synthesis system using different energy sources. The plasmid, pk7EPO, was incubated in a reaction mixture containing PEP, CP, or 3PG as an energy source for ATP regeneration. Ten microliter reaction samples were withdrawn at the indicated time points in order to measure the amounts of expressed protein (filled circle) and ATP (open circle) as described in Materials and methods. Insets, after 150 min of incubation, 2 μl of the reaction mixture was isolated and analyzed by Western blot analysis.

the effect of the nucleotide sequence on the efficiency of termination varies depending on the environment of the protein synthesis reactions.

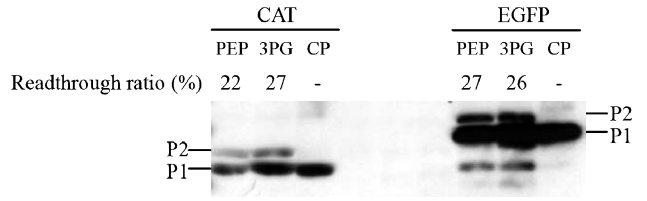

Fig. 4. Readthrough of CAT and EGFP in a cell-free protein synthesis system using different energy sources. After 2 h of incubation, 2 μl of the reaction mixture was loaded on Tricine–SDS–PAGE and analyzed by Western blot. The readthrough ratio observed in each lane was determined by dividing the amount of readthrough product by the total amount of expressed protein. P1 and P2 represent the correctly terminated and readthrough hEPO, respectively.

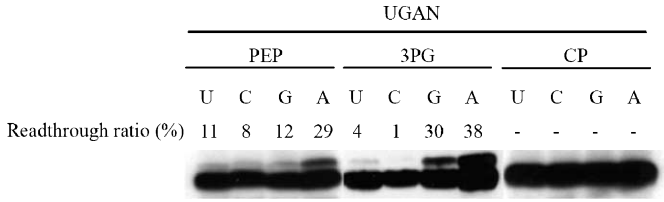

Fig. 5. Effect of the nucleotide context on the readthrough of the UGAN stop codon in three energy regeneration systems (PEP, 3PG, and CP). The fourth base is observed in each lane. After 2 h of incubation, 2 μl of the reaction mixture was isolated and analyzed by Western blot analysis. The readthrough ratio observed in each lane was determined by dividing the amount of readthrough product by the total amount of expressed protein.

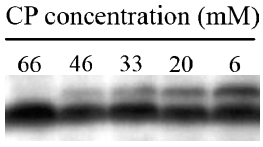

Fig. 6. Effect of CP concentration on the readthrough efficiency in a cell-free protein synthesis.

Surprisingly, regardless of the identity of the +4 nucleotide, virtually all of the translation products were found to be of the expected size in the CP-based reactions. On the other hand, in the reaction using creatine phosphate, a significant amount of readthrough product was detected when the concentration of creatine phosphate was lowered and the efficiency of ATP regeneration was reduced (Fig. 6).

Therefore, the implementation of proper energy regeneration eliminates the need to engineer the nucleotide sequence so as to allow the expression of the properly terminated proteins.

Discussion

One of the major advantages of cell-free protein synthesis is that it offers the possibility of rapidly translating genetic information into protein molecules. Moreover, since the target protein is selectively synthesized in an 'open' system, the completed reactions can be directly used for the subsequent analysis of the expressed

proteins. However, the generation of readthrough products requires an additional step to be performed, in order to separate the target protein of the correct size from the contaminating readthrough products, which detracts from the advantages of cell-free synthesis. Among the three stop codons, the phenomenon of readthrough is most prominent with UGA [9], and our results indicate that the frequency of readthrough is significant during the cell-free synthesis of recombinant proteins. Unfortunately, however, most of the currently available vectors, such as the pET vectors, carry UGA as an in-frame stop codon.

In this study, we discovered that the phenomena of stop codon readthrough are significantly affected by the energy source that is used for the regeneration of the nucleotide triphosphates. PEP, the most widely used energy source in cell-free protein synthesis, charges ADP by the action of pyruvate kinase. 3PG is the 8th intermediate compound of the glycolytic pathway which is converted to PEP (via 2PG) and then used for ATP regeneration [19]. While PEP and 3PG can be utilized by the endogenous enzymes present in the cell-free extract, CP transfers its phosphate group to nucleoside diphosphates in the presence of exogenously added creatine kinase. In addition, CP has an optimal concentration which is higher than that of PEP or 3PG and provides more stable maintenance of ATP. Although the exact mechanism by which the termination efficiency is affected by the energy supply remains to be clarified, our results imply that there is an intimate correlation between the termination fidelity and the efficiency of energy regeneration. The results in Fig. 6 support this hypothesis, given that readthrough of the stop codon occurred in a reaction using CP when its concentration was lowered to reduce the regeneration of NTPs.

It is known that the frequency of readthrough is strongly affected by the identity of the nucleotide situated next to the stop codon [11,20,21]. However, in this study, even when the context around the stop codon was the same, the three energy regeneration systems showed different readthrough levels (Fig. 5). The CP-CK system produced no readthrough protein, whereas the other energy regeneration systems showed substantial readthrough, albeit with slight differences in the readthrough level.

Acknowledgment

This work was partially funded by the Brain Korea 21 program which is supported by the Ministry of Education, South Korea.

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