A cell-free protein synthesis system as an investigational tool for the translation stop processes

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Abstract Using *Escherichia coli* cell-free protein synthesis system and aminoacylated amber suppressor tRNA, we successfully inserted an unnatural amino acid *S*-(2-nitrobenzyl)-cysteine into human erythropoietin. Three different types of translation stop suppression were observed and each of the three types was easily discerned with SDS–PAGE. Optimal conditions were established for correct stop and programmed suppressions. Since this system differentiates proteins produced by misreading of codons from those produced by programmed suppression, we conclude that this cell-free translation system that we describe in this paper will be of a great use for future investigations on translation stop processes. © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

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1. Introduction

In principle, the three codons, UAA, UAG, and UGA, terminate translation, but they can also serve as sense codons in the mitochondrial translation system [1] and be misread or read-through when competing with protein releasing factors. Hence, it is suggested that acting as the signal for termination is not the sole function, but part of the function of these stop codons.

Recently the stop codons have been used as sense codons by devising a tRNA that would suppress the termination function, especially after Chapeville et al. [2] showed that a tRNA bearing a non-cognate amino acid could be used for the elaboration of a modified protein. Since then, much effort has been made in this direction, using the stop codons, to prepare proteins that contain synthetic amino acids at single, predetermined positions.

The strategy involves misacylated tRNA either to suppress nonsense codons in the mRNA [3–8] or to read a group of four bases as a codon (frame shift) at predetermined sites [9] in the mRNA. To generate mis-aminoacylated tRNA, Hecht et al. [3] used T4 RNA ligase to ligate tRNAs with no 3′-terminal dinucleotide and an aminoacylated derivative of pCpA. Preparation of misacylated tRNA has been greatly facilitated by use of pdCpA, instead of pCpA, and in vitro RNA transcripts. Other improvements in the methodology have been achieved by aminoacylation of pdCpA with cyanomethyl ester of unnatural amino acids [5] and by protecting α-amine with a 4-pentenoyl group [6].

Misacylated tRNA has been successfully employed to prepare numerous polypeptides and proteins. Several synthetic amino acids have also been successfully inserted into these polypeptides and proteins [8]. The actual mechanism of both formation of peptides and initiation of translation by this 'unnatural amino acid mutagenesis' was previously investigated [10,11]. The parameters that control the read-through of nonsense codons were also characterized [8].

However, some difficulties may be encountered in using this technique, simply because it is difficult to differentiate natural suppression events from programmed ones. Furthermore, there are no standard analytical methods by which one can classify the nature of an observed suppressed event at a predetermined site of a protein into the categories of misreading, frame shift read-through, or programmed suppression of the stop codons.

Should there be an analytical system which can tell the nature of a suppressed event, the system would serve as a useful investigational tool for the translation stop and the suppression events. To this end, we have devised an unnatural amino acid mutagenesis system where *S*-(2-nitrobenzyl)cysteine was used as an unnatural amino acid and the human erythropoietin (EPO) as a target protein. We report in this paper that we have found all of the above-mentioned translation stop suppression events took place under controlled conditions. Moreover, these events are observable with conventional SDS–PAGE/Western analyses. We conclude that *S*-(2-nitrobenzyl)cysteine/EPO can be used for investigations on the terminal events of translation and its regulatory elements.

2. Materials and methods

2.1. Construction of plasmids

The 38th residue of the human EPO was chosen and replaced by an amber codon. Plasmid pN38am was constructed using the Quick-

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Change™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). pK7-EPO [12] was used as a template for the polymerase chain reaction and P1/P2 as mutagenic primers. pN38am(TGAA) and pN38am(TAATAA) were constructed following the above-mentioned procedures except that P3/P4 and P5/P6 were used as mutagenic primers, respectively. The constructed plasmid was sequenced and used to transform *Escherichia coli* JM109. Plasmid pSup-ala was constructed by ligating hybridized oligo-DNA templates P7 and P8 to *Eco*RI/*Pst*I fragment of pUC19 as described by Karginov et al. [11]. pFull-ala was constructed by inserting an additional two bases (CA) to the 3′-end of the tRNA-coding gene in pSup-ala by site-directed mutagenesis using P9/P10 as mutagenic primers. The sequences of oligonucleotides used are summarized in Table 1.

2.2. Synthesis of the misacylated suppressor tRNA

N-(4-Pentenoyl),*S*-(2-nitrobenzyl)cysteine cyanomethyl ester: in brief, *S*-(2-nitrobenzyl)cysteine was prepared from the reaction of cysteine hydrochloride (Bachem AG, Bubendorf, Switzerland) and 2-nitrobenzyl chloride (Aldrich, Milwaukee, WI, USA) in a triethylamine-dissolved solution (ethanol:water, 3:5 v/v). The amine residue of *S*-(2-nitrobenzyl)cysteine was protected as the *N*-(4-pentenoyl) derivative [13] and *N*-(4-pentenoyl),*S*-(2-nitrobenzyl)cysteine was activated as the cyanomethyl ester [5].

N-(4-Pentenoyl),*S*-(2-nitrobenzyl)cysteine-pdCpA: the dinucleotide pdCpA (as a tetrabutylammonium salt, Cruachem Inc., Dulles, USA) was aminoacylated with *N*-(4-pentenoyl),*S*-(2-nitrobenzyl)cysteine cyanomethyl ester as described by Robertson et al. [5].

2.3. Construction of suppressor tRNA

Both full-length tRNA and tRNA(−CA) with no 3′-terminal CA were synthesized by run-off transcription of the *Fok*I fragment of pFull-ala and pSup-ala, respectively, from RiboMAX™ large scale RNA production system (Promega, Madison, WI, USA). Crude transcripts were separated on PAGE and bands corresponding to the expected sizes were excised. After full-length tRNA and tRNA(−CA) were extracted, *N*-(4-pentenoyl),*S*-(2-nitrobenzyl)cysteine-pdCpA was ligated to tRNA(−CA) by T4 RNA ligase. A typical 200 μl reaction mixture contained 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 0.02% bovine serum albumin, 100 μg tRNA(−CA), 1 mM acyl pdCpA, 10% (volume) DMSO, and 250 U T4 RNA ligase (Takara Shuzo, Otsu, Shiga, Japan). Ligation efficiency was highest when incubated at 4°C for 12 h. Crude ligate was extracted twice with phenol:chloroform:isoamylalcohol (1:1:1 v/v) and precipitated with three volumes of ethanol. The 4-pentenoyl protecting group at the α-amine moiety of amino acid was removed by iodine as described [6]. One part of iodine solution in THF:water (1:1 v/v) was mixed with four parts of tRNA solution, and left for 30 min at room temperature. tRNA was precipitated with three volumes of ethanol and used immediately or stored at −70°C.

2.4. Cell-free translation and suppression reaction

Cell-free translation and its suppression by *E. coli* S30 were carried out as described [12] with minor modifications. Concentrations of Mg^{2+} were adjusted to be optimal for each and every batch of DNA, suppressor tRNA, and S30.

2.5. Western blot analysis

Samples separated on SDS–PAGE or SDS–urea–PAGE were transferred to nitrocellulose membrane. The wild-type EPO and the mutein were detected through a sequential reaction of polyclonal anti-human EPO (Sigma, St. Louis, MO, USA, 1/2000 in Tris-buffered saline with 0.1% Tween-20), horseradish peroxidase-conjugated anti-rabbit IgG (Sigma, 1/2000 Tris-buffered saline with 0.1% Tween-20), and enhanced chemical luminescence.

3. Results and discussion

There are three possible ways to achieve the suppression of translation stop signals. The first way is through misreadings of codons where ribosomes adopt a non-cognate tRNA as a decoder of the stop signals. The second is by shifting the reading frame in which the ribosomes skip a base or re-read the 3′-base of the previous codon. The last is to employ suppressor tRNAs in the translation process. In the case of misreading, products have roughly similar sizes, and are likely microheterogenous. However, the suppression products by frame shifts are variable in size. On the other hand, employment of tRNAs has been suggested as a potential method of a human gene therapy for somatic diseases [14].

Mutein of EPO was expressed in the presence of suppressor tRNA. Changes in electrophoretic mobility of the expressed protein were followed as the amount of suppressor tRNA increased. When the translation mixture was analyzed with Western blot, the unnatural amino acid was found in the protein (Fig. 1A) and observed were all of the three possible suppressions; background suppression caused by misreading at the 38th codon (amber stop codon), as shown in Fig. 1A, lane 1, suppression by the frame shift also occurred, and downstream termination of translation by the authentic stop codon. However, the last produced EPO which is larger than the wild-type EPO (lanes 6 and 7, Fig. 1A). Finally, the synthesis of mutein with the unnatural amino acid was initiated only by an addition of the supplemented suppressor tRNA (lanes 3–5, Fig. 1A). Moreover, with an increase in the amount of suppressor tRNA in the translation reactions, the programmed suppression became more dominant over background suppression. When 0.4 μg of the suppressor tRNA was used, practically no background suppression was observed (lane 5, Fig. 1A). This observation can be explained by the fact that ribosomes favor ternary complex, cognate aminoacyl-tRNA·EF-Tu·GTP, in the translation. Apparently, cognate tRNA (suppressor tRNA in our experiment) is kinetically favored [15].

Unexpectedly, synthesized mutein moved faster on PAGE than either the wild-type EPO or the protein synthesized by background suppression did. This was also the case with the frame shift products of the programmed mutein. This phenomenon was observed throughout our studies, regardless of variables in the reducing power of PAGE sample buffers or the heat denaturing procedures. On the contrary, both products of the background suppression and its frame shift showed the same mobility as that of the wild-type EPO and its frame shift product. Incorporation of *S*-(2-nitrobenzyl)cysteine into the 38th residue of EPO might have caused the difference in mobility of these proteins.

Conformational or electrostatic changes of protein can also alter its electrophoretic mobility [16]. SDS–urea–PAGE was carried out to see if the observed changes in the mobility of mutein EPO, and their respective frame shift products are due to conformational or electrostatic changes. Since mutein and its frame shift product on SDS–urea–PAGE (Fig. 1B) have the same mobility as wild-type EPO and its frame shift products, as compared to SDS–PAGE, we conclude that *S*-(2-nitrobenzyl)cysteine was successfully incorporated into EPO and that the resulting EPO is different from the wild-type EPO with regard to physical properties like conformation, electrostatic nature, or solubility.

The mutein produced by the unnatural amino acid mutagenesis might contain a microheterogenous structure by the action of suppressor tRNA charged with one of the 20 natural amino acids. This may be the consequence of the deaminoacylation of synthetically attached artificial amino acid from suppressor tRNA and reaminoacylation with one of the 20 natural amino acids existing in the translation system. However, full-length suppressor tRNA with no aminoacylation did

Table 1
Oligonucleotide sequences for the construction of plasmids used in this study

Oligonucleotide	Orientation	Sequence (5′ to 3′)	Plasmid
P1	Sense	GAACACTGCAGCTTGAATGAGTAGATCACTGTCCCA	pN38am
P2	Antisense	Complementary to P1	
P3	Sense	GGGGACAGATGAAGTCGACCGGCTGC	pN38am(TGAA)
P4	Antisense	Complementary to P3	
P5	Sense	GGGGACAGATAATAAGTCGACCGGCTGC	pN38am(TAATAA)
P6	Antisense	Complementary to P5	
P7[a]	Sense	AATTCTAATACGACTCACTATAGGGGCTATAGCTCAGCTGGGAGAGCGCTTGCATCTAAAGCAA GAGGTCAGCGGTTCGATCCCGCTTAGCCCCACCAGGATCCGGATCCTTCTGCA	pSup-ala
P8[a]	Antisense	GAAGGATGCGGATCCTGGTGGGGCTAAGCGGGATCGAACCGCTGACCTCTTGCTTTAGATGC AAGCGCTCCCCAGCTGAGCTATAGCCCCTATAGTGAGTCGTATTAG	
P9	Sense	CCCGCTTAGCCCCACCACAGGATCCGCATCC	pFull-ala
P10	Antisense	Complementary to P9	

[a]P7 and P8 were 5′-phosphorylated.

not give the suppression product (Fig. 2). As confirmed in Fig. 2, this was not the result of the inhibitory effect of the tRNAs on the translation. Hence, mutein synthesized in our system is free from contaminating microheterogenity.

In the sense of the production of proteins containing unnatural amino acid, the background suppression and the frame shift suppression of nonsense codon would be eliminated. Background suppression can be minimized by an addition of suppressor tRNAs to the translation system more than the threshold amount as depicted in our present study. On the other hand, frame shift suppression can be prevented by changing mRNA (thus DNA) sequences downstream of the stop codon. Several combinations of nucleotide sequences downstream of the stop codon and stop codons themselves were reported for the prevention of frame shift suppression [17,18]. We previously demonstrated that a three-nucleotide stop codon may not be enough to terminate the protein synthesis, and +1 neighboring nucleotide was highly biased for correct termination [17]. The most effective nucleotide is A and four-nucleotide stop codon TGAA is even more efficient than double stop codon (TGATGA). Later, Macmillan et al. [18] showed that the read-through by frame shift occurs in the same vector (pET-16b)/cDNA/TGA stop codon system. The researchers solved the situation by introducing another stop codon adjacent to the 5′ to original stop codon (TGA to TAATGA). However, all those reported previously were examined in an in vivo expression system where conditions for the transcription/translation of target gene cannot be easily manipulated. On the contrary, in the cell-free translation system, it is easier than in the in vivo system to change the parameters for the transcription and translation of a certain gene. We initially searched for the optimal translation conditions, and the search was focused on the concentration of magnesium acetate in the reaction and the sources of S30 preparations. The frame shift suppression was affected by the salt concentrations in the reaction mixture or the sources of S30 preparations, but could not be totally inhibited (lane 1 in Fig. 3 vs. lane 5 in Fig. 1). Moreover, suppression efficiency at the predetermined site could also be affected by this approach especially by the concentration of the magnesium ion. Thus, we reconstructed plasmid pN38am by modifying the downstream of stop codon (TGA to TGAA) or by changing

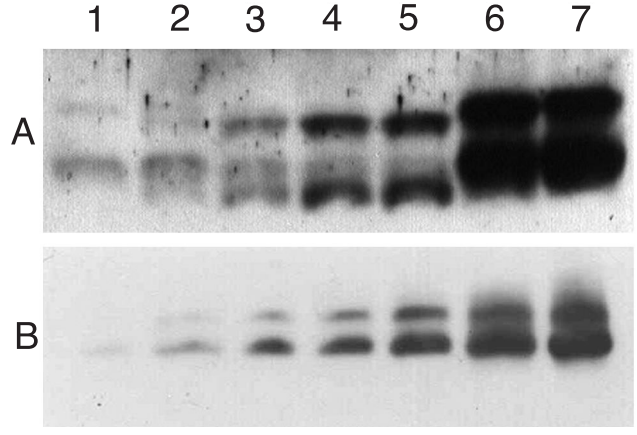

Fig. 1. Western blot analysis of reaction mixtures. Cell-free reaction mixture (2 μl from 15 μl reactions) was applied to (A) 12% SDS–PAGE or (B) 12% SDS–urea–PAGE, and analyzed by Western blot. Reactions contained: lane 1, 0.1 μg pN38am only; lanes 2–5, 0.1 μg pN38am with 0.5, 1, 2, and 4 μg suppressor tRNA charged with *S*-(2-nitrobenzyl)cysteine, respectively; lanes 6 and 7, 0.1 μg pK7-EPO only. Fast moving bands on lanes 6 and 7 correspond to wild-type EPO.

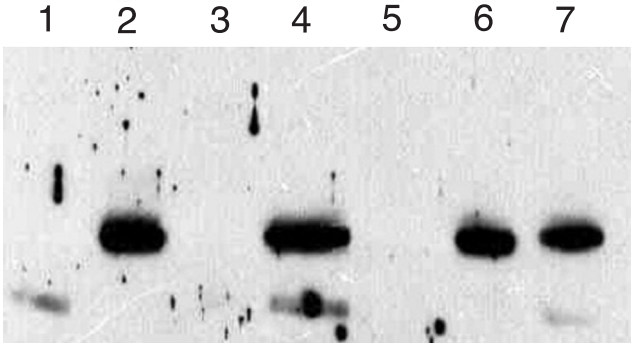

Fig. 2. Re(de)aminoacylation of suppressor tRNA was not detected. Cell-free reaction mixture (2 μl from 15 μl reactions except for lane 2, where 0.5 μl sample was used) were applied to 12% SDS–urea–PAGE and analyzed by Western blot. Reactions contained: lane 1, no DNA; lane 2, 0.1 μg pK7-EPO; lane 3, 0.1 μg pN38am(TAATAA) only; lane 4, 0.1 μg pN38am with 4 μg suppressor tRNA charged with *S*-(2-nitrobenzyl)cysteine; lane 5, 0.1 μg pN38am-(TAATAA) with 4 μg non-charged full-length suppressor tRNA; lane 6, 0.1 μg pK7-EPO with 4 μg suppressor tRNA charged with *S*-(2-nitrobenzyl)cysteine; lane 7, 0.1 μg pK7-EPO with 4 μg non-charged full-length suppressor tRNA. Suppressor tRNAs (either charged or not) did not affect the translation of wild-type EPO.

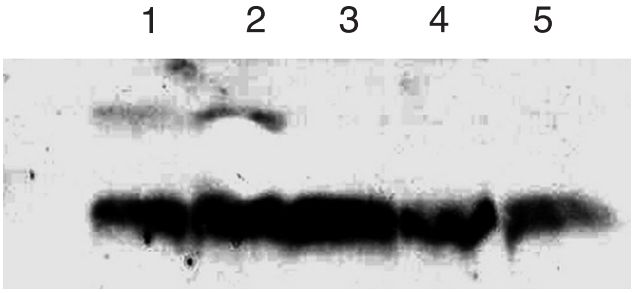

Fig. 3. Stop codon dependence of the frame shift suppression of translation stop. Cell-free reaction mixture (2 μl from 15 μl reactions) was applied to 13.5% SDS–PAGE and analyzed by Western blot. The reactions contained the following: lane 1, 0.1 μg pN38am with suppressor tRNA; lane 2, 0.1 μg pN38am(TGAA) with suppressor tRNA; lanes 3–5, 0.1, 0.2, 0.3 μg pN38am(TAATAA) with suppressor tRNA, respectively. In the case of a programmed suppression reaction, 4 μg of suppressor tRNA charged with *S*-(2-nitrobenzyl)cysteine was used. Samples on lanes 1 and 2 were produced under optimized conditions (S30 prepared from *E. coli* A19, 16 mM Mg^{2+}, 60 mM K^{+}).

the stop codon itself (TGA to TAATAA) using site-directed mutagenesis as described in Section 2. Compared to original pN38am, pN38am(TAATAA) gave more correct termination but pN38am(TGAA) failed to achieve the correct termination (Fig. 3). When a concentration of pN38am(TAATAA) was increased, though no frame shift product was observed, it showed the decrease of the protein band intensity. The amounts of [^{14}C]leu incorporated into the synthesized protein were constant among the samples loaded on lanes 3–5 (data not shown). Thus, the efficiency of the programmed suppression seemed dependent on the concentrations of pN38am-(TAATAA).

However, this result does not agree with our earlier findings, as TGAA could not exactly stop the protein synthesis in the present experiments. This discrepancy may have resulted from differences in protein synthesizing methods (in vitro vs. in vivo). The differences may lie in the availability of factors regulating the translation stop or the diversity of cofactors recognizing different four-nucleotide stop codons. As far as a translation system is concerned, there is a need for a convenient system that classifies well the translation stop, stop suppression, and regulatory factors for both.

Preferably, it will be desirable if that system discriminates all possible unnecessary events related to translation stop, i.e. misreading, programmed suppression and frame shift read-through. Thus, our model system is useful for the investigation of the translation stop processes.

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