A new translational elongation factor for selenocysteyl-tRNA in eucaryotes

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Abstract In eucaryotes, selenocysteine (SeCys) was found in some selenoproteins, but SeCys-tRNA was not recognized by EF-1α. A different translational elongation factor for SeCys-tRNA must therefore supply SeCys-tRNA to the machinery of selenoprotein translation. I found this factor in bovine liver extracts with a UGA-programmed ribosome binding assay. The activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes was eluted in fractions 57–65 using a CM-Sephadex C-25 column, and separated from EF-1α (the activity of binding of [^{14}C]Phe-tRNA to the UUU-programmed ribosomes) in fractions 25–37. EF-1α in fraction 25 could discriminate $(UUU)_{10}$ for [^{14}C]Phe-tRNA. A factor in fraction 57 could discriminate $(UGA)_{10}$ for [^{75}Se]SeCys-tRNA. The elution pattern of activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes was almost identical to that of activity of protecting [^{75}Se]SeCys-tRNA against alkaline hydrolysis (SePF activity) [FEBS Lett. 347 (1994) 137–142]. These two activities might depend on the same factor. The activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes directly indicates that a factor in fraction 57 is a new translational elongation factor for SeCys-tRNA in eucaryotes.

Key words: Translational elongation factor; Selenocysteine; tRNA; EF-1α

1. Introduction

Selenocysteine (SeCys) is an unusual amino acid, found in some proteins such as glutathione peroxidase (GSHPx) [1], type-1 iodothyronine deiodinase [2], and selenoprotein P [3] in mammals. SeCys shares a UGA codon with a stop codon, and a $tRNA^{SeCys}$ for decoding UGA to SeCys was identified [4]. With amino acid codon and tRNA, SeCys is translated on the ribosomes. Therefore SeCys is regarded as the twenty-first amino acid. We showed the presence of SeCys-tRNA synthase and selenophosphate synthetase in bovine liver extracts [5]; these enzymes are also found in *Escherichia coli* [6].

The translational elongation of ribosome-bound polypeptides in eucaryotes requires two complementary factors, EF-1 [7] and EF-2 [8]. EF-1 catalyzes the binding of aminoacyl-tRNA to the ribosomes, and is composed of varying amounts of EF-1α and EF-1βγ [9], while EF-2 translocates the peptidyl-tRNA from the aminoacyl site to the peptidyl site. Among the EF-1 constituents, binding of the aminoacyl-tRNA to the ribosomes is dependent on EF-1α only. In eucaryotes, it is believed that EF-1α is the only factor which can recognize aminoacyl-tRNA and bind it to ribosomes.

In a previous paper [10], my coworkers and I reported that EF-1α from *Bombyx mori* could not protect [^{75}Se]SeCys-tRNA against alkaline hydrolysis. This meant that EF-1α could not recognize [^{75}Se]SeCys-tRNA as the substrate, and could not act as the translational elongation factor for SeCys-tRNA, though SeCys was translated on the ribosomes. Then I searched for the translational elongation factor for SeCys-tRNA in bovine liver extract with an assay of protecting of [^{75}Se]SeCys-tRNA against alkaline hydrolysis, and found in different fractions from those which contained EF-1α. This factor could recognize [^{75}Se]SeCys-tRNA and protect it against alkaline hydrolysis. Although the activity of protecting aminoacyl-tRNA against alkaline hydrolysis is a function of a translational elongation factor, it is not enough to regard this factor as the translational elongation factor for SeCys-tRNA with only protecting activity.

In this study, in order to search for the translational elongation factor for SeCys-tRNA, I developed an mRNA-programmed ribosome binding assay. In this assay, the amount of labeled aminoacyl-tRNA bound to mRNA-programmed ribosomes on the membrane is measured, the binding function of a translational elongation factor is indicated directly. This binding function is the specific function of a translational elongation factor. This assay with [^{14}C]Phe-tRNA and UUU-programmed ribosomes is a standard assay for EF-1α [11]. Then I used [^{75}Se]SeCys-tRNA and UGA-programmed ribosomes to search for the translational elongation factor for SeCys-tRNA, and found it in bovine liver extracts. It was separated from EF-1α by CM-Sephadex C-25 column chromatography, and was eluted in the same fractions as those which contained SePF [10].

2. Materials and methods

2.1. Materials for mRNA-programmed ribosome binding assay

This asay requires three components, labeled aminoacyl-tRNA, 80S ribosomes, and mRNA. These components were prepared as follows.

2.1.1. Preparation of [^{14}C]Phe-tRNA and [^{75}Se]SeCys-tRNA. [^{14}C]Phe-tRNA: $tRNA^{Phe}$ was prepared from bovine liver, and phenylalanylation was performed with partially purified bovine PheRS, according to methods described in detail in [10]. [^{14}C]Phe-tRNA was separated from free [^{14}C]phenylalanine by gel filtration using a Sephacryl S-200 column.

[^{75}Se]SeCys-tRNA: $tRNA^{SeCys}$ was prepared from bovine liver according to methods described in [12]. Selenocysteylation was performed as follows and described in detail in [10]. SerRS was purified from bovine liver [13]. [^{75}Se]HSe^- was prepared from [^{75}Se]selenite by the enzymatic methods of Ganther [14]; the final specific radioactivity of the [^{75}Se]HSe^- was 1.6 Ci/mmol (6.0 μmol). The [^{75}Se]SeCys-tRNA obtained according to the method of [10] contained [^{75}Se]HSe^- and added enzymes. In the presence of these contaminants the values of

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Abbreviations: SeCys, selenocysteine; SePF, SeCys-tRNA protecting factor; SerRS, seryl-tRNA synthetase; PheRS, phenylalanyl-tRNA synthetase.

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mRNA-programmed ribosome binding assay could not be measured precisely, because of high background. In order to remove these contaminants, some treatments were performed as follows. The crude [^{75}Se]SeCys-tRNA precipitate was dissolved in 40 μl of each treatment solution, mixed vigorously for 5 min. Then [^{75}Se]SeCys-tRNA was reprecipitated with ethanol. In the treatment with buffering phenol, the crude [^{75}Se]SeCys-tRNA precipitate was dissolved in 40 μl of distilled water and the equivalent volume of buffering phenol was added. After mixing vigorously for 5 min, the tube was centrifugated for 5 min at 2,000 × g. The upper layer was recovered carefully. Then the [^{75}Se]SeCys-tRNA was reprecipitated with ethanol just as in the other treatments. The supernatants were removed carefully, and then the recovered [^{75}Se]SeCys-tRNA was hydrolyzed with a small volume of 2.5% NH_4OH for 30 min at 37°C and the tRNA was reprecipitated with ethanol. The supernatant which contained the [^{75}Se]SeCys hydrolyzed from [^{75}Se]SeCys-tRNA was immediately analyzed by TLC on a silica-gel G plate developed with (n-butanol/acetic acid/water, 4:1:1). After development, the [^{75}Se]SeCys and other contaminants were detected with a Bioimage Analyzer BAS2000 (Fujix). The contaminants such as [^{75}Se]HSe^- were removed from the above crude [^{75}Se]SeCys-tRNA precipitates with buffering phenol (Fig. 1).This purified [^{75}Se]SeCys-tRNA treated with buffering phenol was used for the mRNA-programmed ribosome binding assay and assay of the activity of protecting [^{75}Se]SeCys-tRNA against alkaline hydrolysis (SePF activity).

2.1.2. Preparation of 80S ribosomes. 80S ribosomes were prepared from bovine brains according to the method of Weissbach [15]. These 80S ribosomes were relatively free of endogenous mRNA and transfer factors.

2.1.3. Preparation of mRNA. mRNA analogues: $(UUU)_{10}$ was purchased from Sigma, and $(UGA)_{10}$ was synthesized with a DNA/RNA synthesizer 392 (Applied Biosystems), and the product was confirmed by 7 M urea PAGE.

2.1.4. mRNA-programmed ribosome binding assay. mRNA-programmed ribosome binding assay was performed according to the generally used method of measuring EF-1α activity [11], with some modifications. The standard assay mixture (final volume of 50 μl) contained 50 mM Tris-HCl (pH 7.4), 75 mM KCl, 5 mM $Mg(CH_3COO)_2$, 0.2 mM DTT, 0.1 mM GTP, 5 μg BSA, 2.5 mM spermidine, 0.5 A_{260} units of 80S ribosomes, 5 μg mRNA analogue, labeled aminoacyl-tRNA, and an appropriate amount of each fractionated protein. Reaction was performed for 10 min at 24°C, and terminated by the addition of 3 ml of cold wash buffer which contained 20 mM Tris-HCl (pH 7.5), 5 mM $Mg(CH_3COO)_2$, and 100 mM NH_4Cl. Immediately after the addition of the cold wash buffer, the reaction mixture was poured onto a nitrocellulose membrane filter (pore size 0.45 μm, Millipore). The inner diameter of the filtration funnel was 8 mm. The membrane was washed twice with 3 ml of cold wash buffer and dried. The radioactivity of the membrane was measured with a Bioimage Analyzer BAS2000 (Fujix).

2.2 Assay of activity of protecting [^{75}Se]SeCys-tRNA against alkaline hydrolysis (SePF activity)

The SePF activity assay was performed according to the method described in [10] with some modifications. In this study, the contaminants were removed with the buffering phenol treatment (Fig. 1). Thus using [^{75}Se]SeCys-tRNA treated with buffering phenol, the step of separation by TLC could be omitted. The protected [^{75}Se]SeCys was spotted on the filtration paper directly, and its amount was measured with a Bioimage Analyzer BAS2000 (Fujix). The measurement errors of the protected [^{75}Se]SeCys spotted on the filtration paper were less than those on the TLC.

2.3. Chromatography condition

Proteins were prepared from microsomes of bovine liver extracts collected by centrifuge at 150,000 × g, as described in [10]. Preliminary experiments showed that microsomes were better for preparation of protein which contained the activity of binding of [^{75}Se]SeCys-tRNA to UGA-programmed ribosomes than cytosol because the level of activity was in microsomes higher than in cytosol.

CM-Sephadex C-25 column (3 × 30 cm) chromatography was performed with a standard buffer composed of 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 10% (v/v) glycerol. The elution was performed with 0.0–0.8 M KCl (linear gradient). The volume of each fraction was 10 ml. Chromatography and preparation were carried out in a cold room at 4°C.

3. Results

First, the contaminants were removed from the [^{75}Se]SeCys-tRNAprecipitate by various treatments; an autoradiograph of TLC plate is shown in Fig. 1. In the assay of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes with the contaminants ([^{75}Se]HSe^- and unknown product at the origin), the values obtained in this assay were inaccurate, because of high background. The contaminants were removed by the treatment with buffering phenol. After the treatment with buffering phenol, the ^{75}Se radioactivity in the [^{75}Se]SeCys-tRNA mixture could be regarded as that of [^{75}Se]SeCys-tRNA only. Thus for the mRNA-programmed ribosome binding assay and assay of the activity of protecting [^{75}Se]SeCys-tRNA against alkaline hydrolysis (SePF activity), [^{75}Se]SeCys-tRNA treated with buffering phenol was used.

In order to search for the translational elongation factor for [^{75}Se]SeCys-tRNA in bovine liver extracts, I developed the mRNA-programmed ribosome binding assay. The codon of SeCys is UGA, which is usually used as a stop codon. Therefore the search for the translational elongation factor for

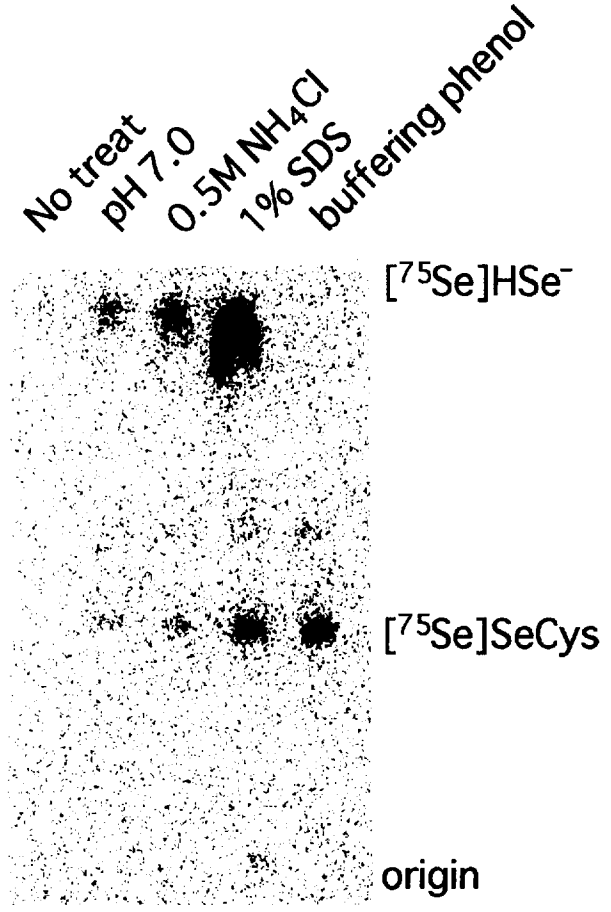

Fig. 1. Separation of [^{75}Se]SeCys-tRNA from [^{75}Se]HSe^- and other contaminants. An autoradiograph of a TLC plate is shown. No treat, negative control; pH 7.0, crude [^{75}Se]SeCys-tRNA precipitate was dissolved in Tris-HCl (pH 7.0); 0.5 M NH_4Cl, crude [^{75}Se]SeCys-tRNA precipitate was dissolved in Tris-HCl (pH 7.0) with 0.5 M NH_4Cl; 1% SDS, crude [^{75}Se]SeCys-tRNA precipitate was dissolved in 1% (w/v) SDS solution; buffering phenol, crude [^{75}Se]SeCys-tRNA precipitate was dissolved in distilled water and treated with buffering phenol (pH 7.0).

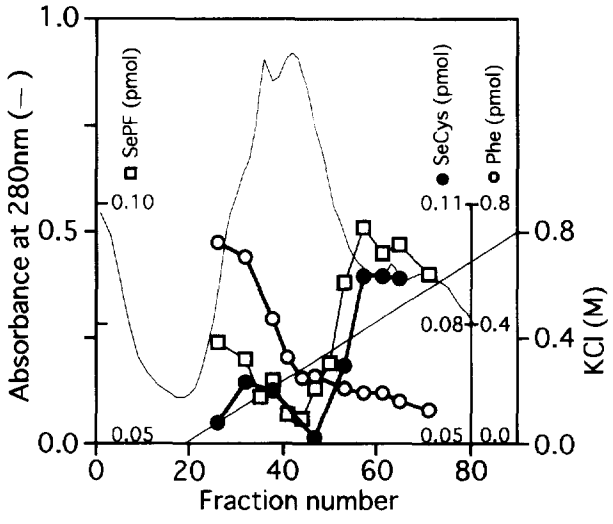

Fig. 2. A chromatographic pattern of extracts from bovine liver microsomes using a CM-Sephadex C-25 column. The activity of binding of [^{14}C]Phe-tRNA to the UUU-programmed ribosomes is shown as open circles (pmol). The activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes is shown as closed circles (pmol). SePF activity which is the activity of a factor protecting [^{75}Se]SeCys-tRNA against alkaline hydrolysis is shown as open squares (pmol). The pass-through fractions, which did not contain these three activities, are omitted.

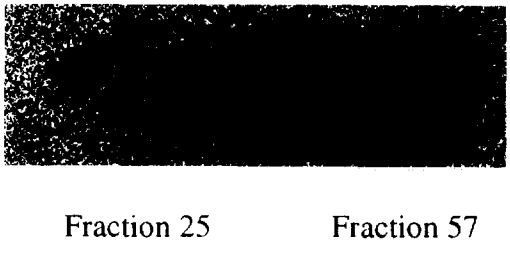

Fig. 3. Autoradiograph of [^{75}Se]SeCys-tRNA bound to the UGA-programmed ribosomes on membranes of fractions 25 and 57. Assays were done in duplicate and the values varied less than 15%.

[^{75}Se]SeCys-tRNA was performed with the UGA-programmed ribosome binding assay. Proteins extracted from bovine liver microsomes were chromatographed using a CM-Sephadex C-25 column, and the results are shown in Fig. 2 (passed-through fractions are omitted). The activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes (closed circles) was found in fractions 57–65. This activity was not found in the passed-through fractions. The autoradiograph of [^{75}Se]SeCys-tRNA bound to the UGA-programmed ribosomes on membranes of fraction 25 and 57 is shown in Fig. 3. The assays were done in duplicate and the amount of [^{75}Se]SeCys-tRNA bound to the UGA-programmed ribosomes varied less than 15%.

On the other hand, the activity of binding of [^{14}C]Phe-tRNA to the UUU-programmed ribosomes (open circles in Fig. 2) was found in fractions 25–37. This assay is a standard assay for EF-1α which is known as the translational elongation factor for all twenty amino acids [11]. The activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes and that of binding of [^{14}C]Phe-tRNA to the UUU-programmed ribosomes were found in distinct fractions. Thus, these two activities were clearly separated from each other by the chromatography. In Fig. 2, the weak activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes in fraction 25–37 may depend upon abundant EF-1α, which may have little affinity to SeCys-tRNA.

The effect of the concentration of protein in fractions 25 and 57 on the activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes is shown in Fig. 4. The level of this activity increased linearly with the concentration of protein in fraction 57, but not with that of protein in fraction 25. The activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes was dependent on a factor in fraction 57.

In the previous paper we reported the existence of a factor protecting [^{75}Se]SeCys-tRNA against alkaline hydrolysis (SePF) [10]. The activity of protecting aminoacyl-tRNA against alkaline hydrolysis is a function of a translational elongation factor, and this SePF could recognize [^{75}Se]SeCys-tRNA. Therefore SePF may have bind [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes. I assayed SePF activity in the fractions, obtained in the chromatography (open circles in Fig. 2), and found it in fractions 57–65. The elution pattern of SePF was almost identical to that of the activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes. This strongly suggested that these two activities depended on the same factor.

A translational elongation factor performed codon–anticodon discrimination with GTP hydrolysis. Thus the codon–anticodon discrimination is a function of a translational elongation factor [16]. The effect of $(UUU)_{10}$ and $(UGA)_{10}$ on the activity of binding of [^{14}C]Phe-tRNA to mRNA-programmed ribosomes of EF-1α in fraction 25 is shown in Fig. 5. EF-1α in fraction 25 bound [^{14}C]Phe-tRNA to the UUU-programmed ribosomes but not to the UGA-programmed ribosomes. Fig. 5 shows that the codon–anticodon discrimination was performed by EF-1α in fraction 25.

On the other hand, the effect of $(UUU)_{10}$ and $(UGA)_{10}$ on the activity of binding of [^{75}Se]SeCys-tRNA to mRNA-programmed ribosomes of a factor in fraction 57 is shown in

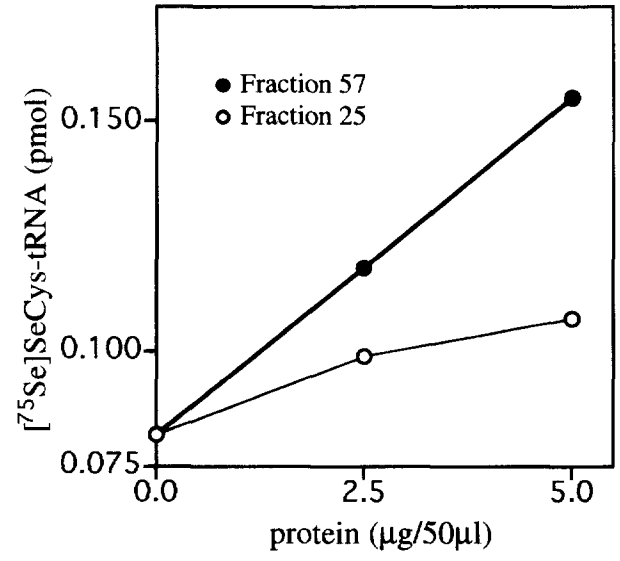

Fig. 4. The effect of concentration of protein in fractions 25 and 57 on the activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes.

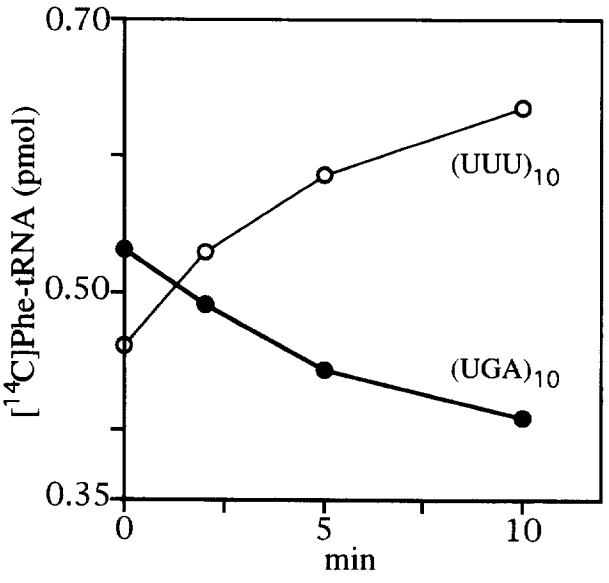

Fig. 5. The effect of $(UUU)_{10}$ and $(UGA)_{10}$ on the activity of binding of [^{14}C]Phe-tRNA to the UUU-programmed ribosomes with fraction 25.

Fig. 6. A factor in fraction 57 bound [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes but not to the UUU-programmed ribosomes. A factor in fraction 57 performed codon anticodon discrimination as did EF-1α in fraction 25.

Table 1 shows a summary of mRNA-programmed ribosome binding assay results. Phe-tRNA was bound by EF-1α in fraction 25, not by protein in fraction 57. SeCys-tRNA was bound by protein in fraction 57, not by EF-1α in fraction 25. In other words, since EF-1α in fraction 25 could not bind SeCys-tRNA, a different translational elongation factor is necessary to bind SeCys-tRNA to the UGA-programmed ribosomes in eucaryotes. A factor in fraction 57 could act as the translational elongation factor for SeCys-tRNA.

4. Discussion

In this study, I found a new translational elongation factor in eucaryotes which bound [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes. This finding is important for the following two reasons.

First, again this is a new translational elongation factor in eucaryotes. A factor in fraction 57 bound aminoacyl-tRNA to mRNA-programmed ribosomes, which is the major function of a translation elongation factor, and performed codon–anticodon discrimination. The elution pattern of activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes in CM-Sephadex C-25 column chromatography was almost identical to that of SePF. These two activities must depend on the same factor. The activity of protecting aminoacyl-tRNA against alkaline hydrolysis and its codon–anticodon discrimination of this factor in fraction 57 strongly suggested that this is a translational elongation factor. Jung et al. reported that SeCys-tRNA was not bound by rabbit EF-1α, and speculated on the existence of a different translational elongation factor, also [17]. The factor in fraction 57 in the present study must be such a factor.

Second, a novel translational elongation factor exists especially for SeCys-tRNA, not for other aminoacyl-tRNA, and EF-1α cannot bind SeCys-tRNA. This second point may be explained by the mechanism of SeCys incorporation to UGA codon for SeCys.

The discrimination of UGA codon for stop or SeCys is a important and interesting problem. In *Escherichia coli*, in which an translational elongation factor (EF-Tu-like) specific to SeCys-tRNA, SELB, has been identified, the information required for UGA-SeCys decoding is present on the stem-loop structure downstream of the UGA codon for SeCys of formate dehydrogenase (FDH) mRNA. SELB has a domain which recognizes this information, and an EF-Tu-like domain. Therefore, fidelity of SeCys on enzyme can only be ensured by SELB in *E. coli* [18].

The factor in fraction 57 might be a counterpart of SELB in eucaryotes. However, the information for UGA-SeCys decoding in eucaryotes is distinct from that of *E coli*. In eucaryotes, the SeCys insertion sequence (SECIS) in the 3′-untranslated region of 5′DI and selenoprotein P have been proposed to be this information [19]. However, this SECIS is located in far from the UGA codon for SeCys, and thus it is difficult to apply the mechanism of UGA-SeCys decoding in *E coli* to that in eucaryotes. The interaction of SECIS and the factor in fraction 57 remains to be investigated.

The factor in fraction 57 is specific to SeCys-tRNA, but it is not clear what determines this specificity, SeCys on tRNA or tRNA itself. SeCys in enzymes plays an important role in catalytic activity; replacement of SeCys residues with other amino acid residues results in a loss of catalytic activity. It was reported that Ser-tRNA^{SeCys} was converted to SeCys-tRNA^{SeCys} by SeCys-tRNA synthase [5]. If this premature Ser-tRNA^{SeCys} was bound to ribosomes by the factor in fraction 57, enzymes with no catalytic activity would be synthesized. Therefore, SeCys not Ser on the tRNA must be required for this specificity. On the other hand tRNA^{SeCys} has unique features, such as 9 base pair (bp) acceptor stem, 6 bp D stem, and 4 bp T stem. This 9 bp acceptor stem is a major identity element to SeCys-

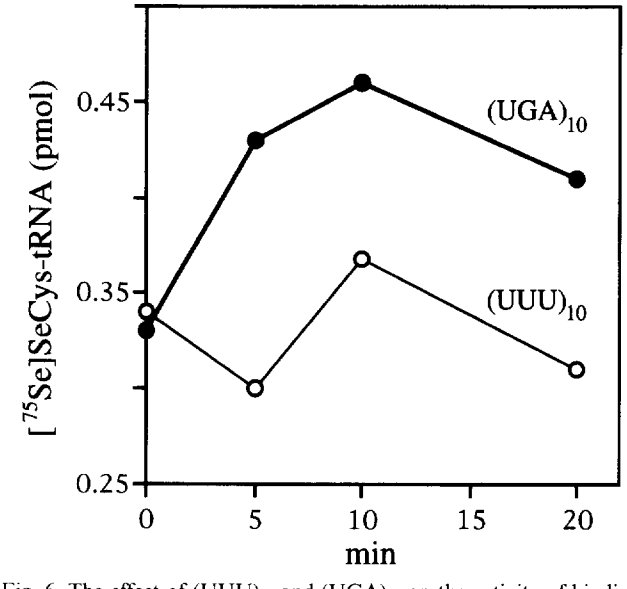

Fig. 6. The effect of $(UUU)_{10}$ and $(UGA)_{10}$ on the activity of binding of [^{75}Se]SeCys-tRNA to the UGA-programmed ribosomes with fraction 57.

Table 1
Summary of mRNA-programmed ribosome binding assay

tRNA	pmol[a]	Codon	pmol bound[b]	
			Fraction 25 (EF-1α)[c]	Fraction 57[c]
Phe-	8.02	UUU	0.75	0.19
SeCys-	0.957	UGA	0.042	0.100

[a] pmol of labeled aminoacyl-tRNA added to each assay.
[b] pmol of labeled aminoacyl-tRNA bound to mRNA-programmed ribosomes for each fraction.
[c] Fraction number of chromatography using CM-Sephadex C-25 column.

tRNA synthase [20]. These unique features may be the determinants of $tRNA^{SeCys}$ to the factor in fraction 57. EF-1α recognizes aminoacylation of the acceptor stem and T arm of tRNA, not the species of amino acid on tRNA. These unique features of $tRNA^{SeCys}$ may be negative determinants for EF-1α.

The finding of a new translational elongation factor suggests that yet others may exist. Recently, two EF-1α isoforms, whose expression are tissue-specific, have been reported [21,22]. Assays for EF-1α have been performed mainly with Phe-tRNA and sometimes with other aminoacyl-tRNAs. Now mRNA-programmed ribosome binding assays can be performed with other aminoacyl-tRNAs more precisely, and other translational elongation factors may be found.

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