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Selenocysteine insertion or termination: factors affecting UGA codon fate and complementary anticodon:codon mutations

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ABSTRACT

Translation of UGA as selenocysteine instead of termination occurs in numerous proteins, and the process of recoding UGA requires specific signals in the corresponding mRNAs. In eukaryotes, stem–loops in the 3′ untranslated region of the mRNAs confer this function. Despite the presence of these signals, selenocysteine incorporation is inefficient. To investigate the reason for this, we examined the effects of the amount of deiodinase cDNA on UGA readthrough in transfected cells, quantitating the full-length and UGA terminated products by Western blotting. The gene for the selenocysteine-specific tRNA was also cotransfected to determine if it was limiting. We find that the concentrations of both the selenoprotein DNA and the tRNA affect the ratio of selenocysteine incorporation to termination. Selenium depletion was also found to decrease readthrough. The fact that the truncated peptide is synthesized intracellularly demonstrates unequivocally that UGA can serve as both a stop and a selenocysteine codon in a single mRNA. Mutation of UGA to UAA (stop) or UUA (leucine) in the deiodinase mRNA abolishes deiodinase activity; but activity is partially restored when selenocysteine tRNAs containing complementary mutations are cotransfected. Thus, UGA is not essential for selenocysteine incorporation in mammalian cells, provided that codon:anticodon complementarity is maintained.

INTRODUCTION

Selenocysteine (Sec) is an unusual amino acid for at least two reasons: (1) it is encoded by UGA which is normally a stop codon; and (2) it is synthesized from serine after serine is conjugated to a specialized tRNA designated $tRNA^{[Ser]Sec}$. Sec is present in numerous prokaryotic and eukaryotic proteins (see 1,2 for reviews and 3–5 for subsequent work), and its presence confers unique biochemical properties on selenoproteins (6,7). Sec-tRNA genes and/or the corresponding gene product have been identified in eubacteria (8), protists (9), fungi and plants (10), and throughout the animal kingdom (11). Due to the widespread occurrence of a Sec-tRNA that decodes UGA in nature, Sec has recently been assigned to UGA in the 'almost universal' genetic code (12).

The process of Sec incorporation into protein is under investigation in both prokaryotes and eukaryotes, and these studies have revealed a number of differences between the two in the decoding process. Translation of UGA in *E.coli* formate dehydrogenase H as Sec requires a short stretch of sequence capable of forming secondary structure which is located adjacent to and immediately downstream of this codon, in the coding region of the mRNA (13). Nuclease sensitivity studies have indicated that this region forms a stable stem–loop, and a specific elongation factor, SELB, has been identified which binds this structure (14). SELB also binds $Sec\text{-}tRNA^{[Ser]Sec}$ (15). No eukaryotic homolog of SELB has yet been identified, although autoantibodies against a Sec-tRNA binding protein have been identified in human sera, suggesting that this protein may be a candidate for such an elongation factor (16). In the eukaryotic selenoproteins, type I iodothyronine deiodinase, glutathione peroxidase, and selenoprotein P, UGA translation requires putative stem–loop structures in the 3′ untranslated regions of the mRNAs (designated SECIS elements), and these elements can be separated from UGA by more than 2 kilobases (17). In eukaryotic mRNAs, a single SECIS element can confer translation of multiple UGA codons (17), whereas this has not been demonstrated in prokaryotes.

In a previous study, we used affinity labeling with the substrate analog, BrAcT3, to quantitate the efficiency of translation of the deiodinase UGA codon in transfected cells (7). We measured expression of the wild-type deiodinase and a mutant in which UGA is replaced by the cysteine codon, UGU, and found that the efficiency of UGA translation ranges from 20- to 400-fold lower than that of UGU, depending on the cell type. We proposed that this low efficiency was due to limiting amounts of the components of the translation machinery essential for decoding UGA as Sec, possibly including $Sec\text{-}tRNA^{[Ser]Sec}$, the putative eukaryotic homolog of prokaryotic SELB, and other

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selenoprotein-specific components of the translation machinery. Additionally, assembly of complexes of these factors and the SECIS elements may be time-limiting.

We are interested in understanding the process of decoding Sec UGA codons. As an initial approach to elucidating this phenomenon, we examined UGA translation efficiency at varying DNA concentrations in transiently transfected cells, since under these conditions, artificially high levels of DNA are used, producing high levels of mRNA, which might exacerbate inefficient translation by saturating the translation machinery. We also investigated whether $tRNA^{[Ser]Sec}$ may be limiting at these high selenoprotein mRNA concentrations, both by cotransfecting the *Xenopus* $tRNA^{[Ser]Sec}$ and wild-type deiodinase genes and by examining the effects of adding the purified tRNA to *in vitro* translation reaction mixtures. Furthermore, we examined the effects of selenium depletion on UGA readthrough and determined the effects of complementary mutations in the UGA codon of deiodinase mRNA and the anticodon of $tRNA^{[Ser]Sec}$.

MATERIALS AND METHODS

Materials

Bovine liver $tRNA^{[Ser]Sec}$ and yeast $tRNA^{Tyr}Su^+$ were purified as described (9). Keyhole limpet hemocyanin and bis-maleimidomethyl ether were from Calbiochem. Horseradish peroxidase-coupled goat anti-rabbit antibody was obtained from Cappel. Enhanced chemilluminescence detection reagents were from Amersham. All other chemicals were of reagent or molecular biology grade.

Constructs

Vectors pUHD10-3 (D10) and pUHD15-1 (D15) were obtained from Dr Manfred Gossen (18). The rat 5′ deiodinase cDNA was cloned into D10 between the *Eco*RI and *Xba*I sites. The resulting deiodinase expression vector was designated G21/D10. The codon 126 UAA (ochre, G3), UUA (leucine, G4), and UGU (cysteine, G5) mutant deiodinase constructs have been described previously (6). All three mutant constructs were subcloned into the D10 vector. The cysteine mutant (G5) was also subcloned into the T7 polymerase-dependent bacterial expression vector, pET-3 (Novagen), and expressed in the BL21(DE3)pLysS strain of *E.coli*. The SECIS loop mutant (RM2) has also been described (17). The mutant stem-loop was substituted for the wild-type stem−loop in G21/D10 and designated RM2/D10. An 800 bp *Hin*dIII−*Eco*RI fragment encoding the wild-type *Xenopus* $tRNA^{[Ser]Sec}$ gene cloned in pGEM-3 (11,19) was used for transfections. The anticodon region was mutated to either UUA or UAA using PCR mutagenesis (20), subcloned back into pGEM-3, and sequenced entirely to confirm that no other mutations were introduced.

Transfections and 5′ deiodinase assays

Human embryonic kidney (HEK) 293 cells were maintained in DMEM + 10% fetal calf serum in the absence of tetracycline. Transfections of wild-type and mutant deiodinase and tRNA-expressing plasmids were performed by $CaPO_4$−DNA precipitation. All dishes received 5 μg D15 plasmid encoding the tetracycline-repressible transactivator (16) and 4 μg TKGH, which constitutively expresses human growth hormone (hGH). Two days after transfection, cells were harvested and sonicated in 0.25 M sucrose, 100 mM sodium phosphate, pH 6.9, 1 mM EDTA and 10 mM DTT. For selenium depletion, cells were cultured in DMEM containing 1% fetal calf serum supplemented with 100 pM 3,5,3′-triiodothyronine (T3), 10 mg/ml transferrin, 20 mg/ml insulin, and 50 nM hydrocortisone, starting 5 days prior to transfection. Paired dishes were each transfected with half of the $CaPO_4$/DNA precipitate mixture, and 50 nM sodium selenite was added to one dish of each pair. For this experiment, four dishes received either G21/D10 and D15 or G21/D10, D15 and the *Xenopus* $tRNA^{Ser]Sec}$ gene. Two of each of the four then received selenium, and the other two did not. Dishes receiving identical treatment were harvested together as described above. 5′ Deiodinase assays were performed as given (6,7) with 1 μM rT3 and 10 mM DTT for 60 min at 37°C. Deiodinase activity is expressed relative to hGH in the media. For each set of transfections and deiodinase assays, positive (G21/D10) and negative (D10 vector) controls were included. All transfections were performed at least twice, and all assays were performed in duplicate.

In vitro translations and immunoprecipitations

In vitro translations were performed with rabbit reticulocyte lysates from Promega (Madison, WI) according to the supplier's instructions. G21 mRNA (0.6 μg per 25 μl reaction) was added to all reaction mixtures. Where indicated, 8 μg of partially purified bovine liver $tRNA^{[Ser]Sec}$ or yeast $tRNA^{Tyr}Su^+$ were added. Rabbit antisera was prepared against a synthetic peptide corresponding to amino acids 45−60 of the rat 5′ deiodinase sequence (see below). Immunoprecipitations were performed with 10 μl translation reaction mixture, 10 μl antisera, and 80 μl PBS at 4°C overnight. Pansorbin (CalBiochem) was added, and pellets washed three times in STN buffer (150 mM NaCl, 10 mM Tris−HCl, pH 8.0, and 0.5% NP40). Immune complexes were resuspended in sample buffer (0.125 mM Tris−HCl, pH 6.8, 2.5% SDS and 0.5 M mercaptoethanol), boiled for 2 min, and electrophoresed on a 15% acrylamide (acrylamide to bis 37.5:1)−SDS gel.

Peptide synthesis

Peptides corresponding to amino acids 45−60, 77−90, 168−183, and 225−240 of the rat 5′ deiodinase sequence, with cysteine as the amino-terminal amino acid, were synthesized on an Applied Biosystems, Inc. Model 430A peptide synthesizer using tBOC chemistry. The first two peptides correspond to sequences that are located on the amino-terminal side of selenocysteine while the latter two peptides are on the carboxy-terminal side of selenocysteine.

Peptide coupling and immunizations

Derivitization of keyhole limpet hemocyanin (KLH) was performed by slow addition of ten 10 μl aliquots of a 25 mg/ml solution of bis-maleimidomethyl ether (BMME) in dimethyl formamide to 2 ml of a 10 mg/ml KLH solution in phosphate-buffered saline (PBS), pH 6.8, while stirring. Derivitized KLH was separated from BMME on a G-25 fine column developed in PBS, pH 6.8. Peptides were dissolved in PBS−20 mM EDTA. For insoluble peptides, small aliquots of concentrated NaOH were added until solubility was obtained. Peptide solutions were slowly added to the derivitized KLH, followed by stirring at r.t. for 3 h, and dialysis against PBS, pH 6.8 at 4°C overnight. Pathogen-free New Zealand White female rabbits, 1.5−2 kg, were

immunized with 300 μg peptide/rabbit in complete Freunds adjuvent divided among four subcutaneous locations. Rabbits were boosted with 600 μg peptide in incomplete Freunds adjuvent at 3–4 week intervals.

Western blotting

Transfected cell sonicate proteins (100–200 μg per lane) were electrophoresed on 13.5% or 15% acrylamide (acrylamide to bis 37.5:1)–SDS gels. Protein gels were electroblotted onto Immobilon (Millipore) in 20% methanol, 25 mM Tris–HCl, pH 8.3, 192 mM glycine at 100 mA overnight at 4°C. Blots were blocked with 5% (w/v) nonfat milk in TBS–Tween (20 mM Tris–HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20). Antisera was used at 1:100 dilution in TBS–Tween. Peptide blocking was carried out by preincubating antisera with a 100-fold molar excess (based on total IgG) of peptide for 1 h at 37°C prior to incubation of antisera with the blot. Horseradish peroxidase-coupled goat anti-rabbit antibody was used at 1:1000 dilution. Enhanced chemilluminescence reagents were used according to the manufacturer's instructions.

RESULTS

Characterization of peptide antisera

Prior to screening antisera against type I 5′ deiodinase, we took advantage of a recently described mammalian expression vector system and the efficiently expressing human embryonic kidney (HEK) 293 cell line to increase the levels of deiodinase expression. The vector system uses a tetracycline-regulatable transactivator (18) to control expression and results in a ~5- to 10-fold increase in deiodinase activity over the levels we had previously obtained with CDM vector in COS cells (6,7). Peptide antisera were screened by Western blotting for reactivity with the transiently transfected wild-type deiodinase (G21/D10) or the UAA or cysteine mutants, with the bacterially expressed cysteine mutant deiodinase (G5), and with hyperthyroid rat liver microsomes. The sequences of peptides 1 and 2, which correspond to amino acids 45–60 and 77–90, respectively, in rat deiodinase, are located upstream of the Sec residue, while peptides 3 and 4, corresponding to amino acids 168–183 and 225–240, respectively, are located downstream of the Sec. Hence, antisera to peptides 1 and 2 would be expected to detect the truncated protein which terminates at the UGA Sec codon as well as the full-length protein. Antisera to peptides 3 and 4, on the other hand, should detect only full-length protein. As shown in Figure 1, antisera against peptides 1, 3, and 4 all detected an ~28 kDa protein in sonicates from HEK 293 cells transfected with the G21 deiodinase clone. The ~28 kDa band corresponds to the full-length deiodinase protein. As expected, antisera to peptides 1, 3, and 4 did not detect an ~28 kDa protein in the D10 vector-transfected cell sonicates (Fig. 1).

The antisera against peptide 1 also reacted with the ~14 kDa protein produced by termination of translation at the UGA Sec codon (Fig. 1, lane 2) and with the 14 kDa protein produced by the UAA mutant (Fig. 1, lane 3). This protein was not detectable in cells transfected with vector alone, nor with antisera against peptides 3 or 4. The specificity of the peptide 1 antisera was confirmed by blocking reactivity with peptide 1 (Fig. 1). Antisera against peptides 1, 3, and 4 also reacted with an ~28 kDa protein in hyperthyroid rat liver microsomes and with

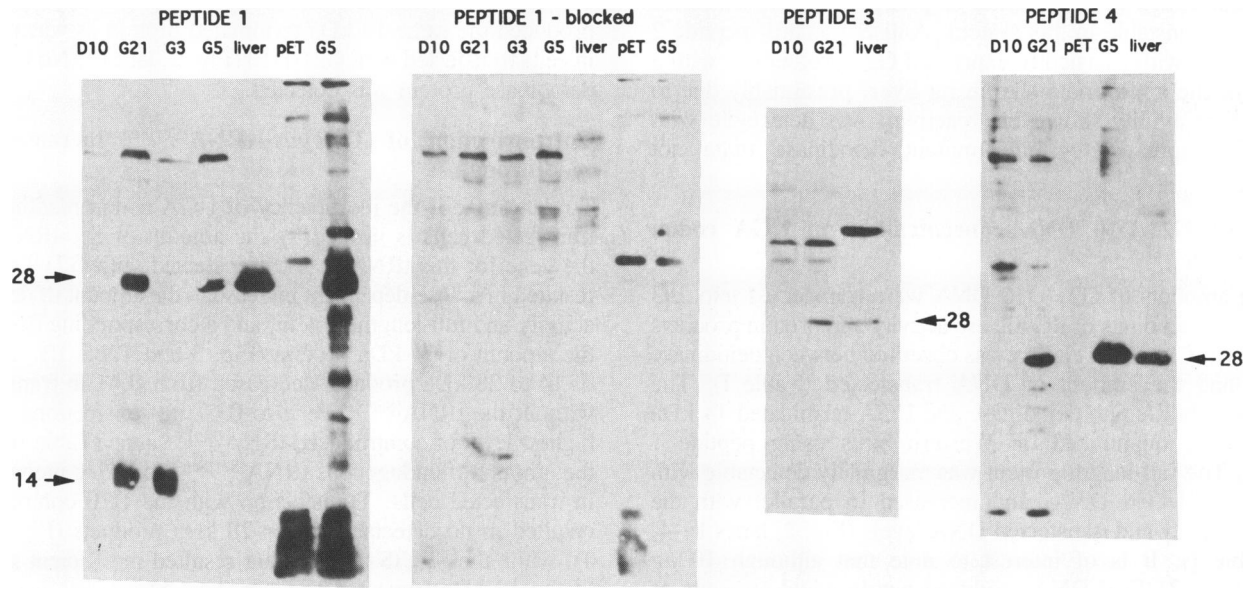

Figure 1. Western blot characterization of anti-deiodinase peptide antisera. Peptide 1, (antisera to amino acids 45–60): lane 1, D10 vector-transfected 293 cells; lane 2, G21/D10-transfected 293 cells; lane 3, UAA mutant G3/D10-transfected 293 cells; lane 4, cysteine mutant G5/D10-transfected 293 cells; lane 5, hyperthyroid rat liver microsomes; lane 6, pET vector-transformed *E.coli*; lane 7, cysteine mutant deiodinase expressed in *E.coli*. Peptide 1-blocked: lanes 1–7, as above except that antisera was preincubated with 100-fold excess of peptide 1 prior to incubation with blot. Peptide 3, (antisera to amino acids 168–183): lane 1, D10 vector-transfected 293 cells; lane 2, G21/D10-transfected 293 cells; lane 3, hyperthyroid rat liver microsomes. Peptide 4, (antisera to amino acids 225–240): lane 1, D10 vector-transfected 293 cells; lane 2, G21/D10-transfected 293 cells; lane 3, pET vector-transformed *E.coli*; lane 4, cysteine mutant deiodinase expressed in *E.coli*; lane 5, hyperthyroid rat liver microsomes. The positions of full-length (28 kDa) and UGA-terminated (14 kDa) proteins are indicated by arrows.

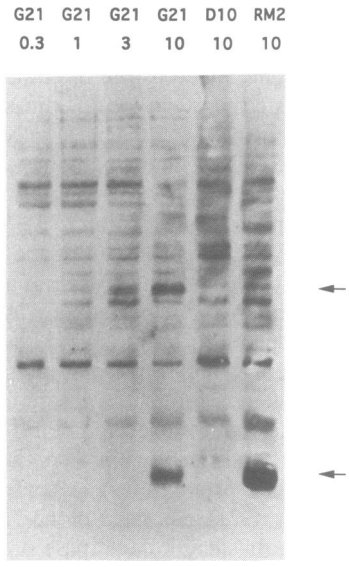

Figure 2. Effects of G21/D10 DNA concentration on UGA codon readthrough. Lane 1, 0.3 μg G21/D10-transfected; lane 2, 1.0 μg G21/D10; lane 3, 3.0 μg G21/D10; lane 4, 10.0 μg G21/D10; lane 5, D10 vector control; lane 6, RM2/D10. Western analysis was performed with peptide 1 antisera. The positions of full-length and UGA-terminated proteins are indicated by arrows.

bacterially-expressed cysteine mutant deiodinase. As expected, no 14 kDa protein was detected in the bacterial extracts, since this mutant does not have the potential for termination at this position, due to the absence of the UGA codon. Interestingly, however, the rat liver microsomes also did not contain any detectable protein of this size, suggesting that all peptides initiated were readthrough to termination or perhaps that the truncated protein was unstable in this system. Antisera against peptide 2 did not react with transiently expressed G21 protein nor with a protein of the appropriate size in rat liver, presumably due to low titer or avidity, however, reactivity was detectable with bacterially-expressed cysteine mutant deiodinase (data not shown).

Effects of G21/D10 DNA concentration on UGA codon readthrough

Varying amounts of G21/D10 DNA were transfected into 293 cells and the amounts of deiodinase activity and protein products quantitated. Close correlation was observed between deiodinase activity and the amount of DNA transfected (Table I). The amounts of full-length deiodinase and UGA-terminated 14 kDa protein were quantitated on Western blots using peptide 1 antisera. The full-length protein was marginally detectable with 0.3 μg transfected DNA, and increased in parallel with the enzyme activity and transfected DNA levels (Fig. 2, lanes 1–4, and Table I). It is of interest to note that although 10 μg transfected G21/D10 DNA produced nearly equal amounts of 14 kDa and full-length protein, no 14 kDa protein was detectable with 0.3–3 μg of transfected DNA, despite the presence of the full-length protein in these homogenates. The control D10 vector resulted in no detectable 14 or 28 kDa products (Fig. 2, lane 5). To verify the identity of the 14 kDa protein and to confirm our previous demonstration that a functional SECIS element is required for translation of UGA (21) we introduced a SECIS loop mutant, RM2, into the G21/D10 expression vector and examined

Table I. Effects of DNA concentration on type I deiodinase activity and on the corresponding protein products

G21-D10 (μg)	Relative activity (c.p.m. I^-/hGH)	14 kDa	28 kDa	14/28kDa ratio
0.3	14	n.d.	37	
1	22	n.d.	61	
3	56	n.d.	122	
10	115	182	203	0.89

DNA concentrations are given as μg plasmid transfected/60 mm plate of 293 cells. Deiodinase activity is expressed as c.p.m. I^- released/μl protein, normalized to c.p.m. hGH released in the media. All transfections contained 5 μg plasmid D15. Quantitation of 14 kDa and 28 kDa proteins was carried out by laser densitometry and is expressed as arbitrary units. n.d., not detectable.

Table II. Effects of *Xenopus* $tRNA^{[Ser]Sec}$ gene cotransfection on type I deiodinase activity and on the corresponding protein products

G21-D10 (μg)	$tRNA^{[Ser]Sec}$ DNA (μg)	Relative activity (c.p.m. I^-/hGH)	14 kDa	28 kDa	14/28 kDa ratio
5	0	89	179	440	0.41
5	5	118	111	523	0.21
5	20	148	49	732	0.07

DNA concentrations are given as μg plasmid transfected/60 mm plate of 293 cells. Deiodinase activity is expressed as c.p.m. I^- released/μl protein, normalized to c.p.m. hGH released in the media. All transfections contained 5 μg plasmid D15. Quantitation of 14 kDa and 28 kDa proteins was carried out by laser densitometry and is expressed as arbitrary units.

the protein products. The mutation consists of a 9 nucleotide deletion, removing the entire loop (21). This mutant efficiently produced the same 14 kDa terminated protein as was observed in cells transfected with G21/D10 (Fig. 2, lane 6). No full-length deiodinase protein was detected.

Cotransfection of *Xenopus* $tRNA^{[Ser]Sec}$ increases UGA readthrough

To determine if the inefficiency of UGA codon readthrough in transfected cells is limited by the amount of Sec-$tRNA^{[Ser]Sec}$, the gene for this tRNA was cotransfected with G21/D10. This resulted in a dose-dependent increase in the amount of deiodinase activity and full-length protein, and a corresponding decrease in the amount of 14 kDa protein (Fig. 3 and Table II). The ratio of 14 to 28 kDa products decreased from 0.41 in transfections without the $tRNA^{[Ser]Sec}$ gene to 0.07 in transfections with the highest level of cointroduced $tRNA^{[Ser]Sec}$ gene (Table II). Thus, the amount of endogenous $tRNA^{[Ser]Sec}$ limits Sec incorporation in transfected cells. Transfection with the D10 control vector resulted in no detectable 14 or 28 kDa products (Fig. 3, lane 4), while the SECIS loop mutant resulted in efficient synthesis of the 14 kDa truncated product (lane 5).

Since we previously observed inefficient UGA readthrough in *in vitro* translation reaction mixtures using reticulocyte lysates (6), we examined the effects of added tRNAs to this system, to determine if supplementing lysates with suppressor tRNA would enhance readthrough. Either purified bovine liver $tRNA^{[Ser]Sec}$ or an opal suppressor tRNA, yeast $tRNA^{Tyr}Su^+$, was added to translation reaction mixtures programmed with G21 mRNA. Both tRNAs enhanced UGA readthrough, resulting in an increase in

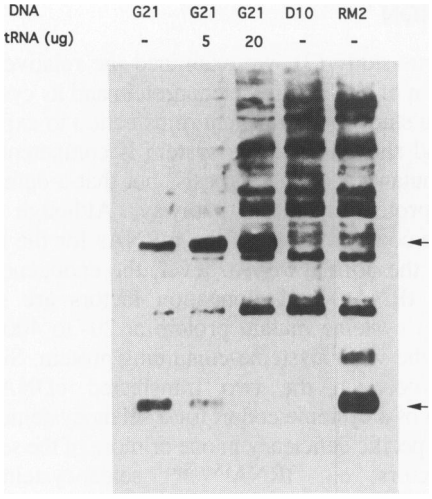

Figure 3. Western analysis of cotransfected G21/D10 DNA and *Xenopus* tRNA$^{[Ser]Sec}$ DNA. Lane 1, G21/D10; lane 2, G21/D10 + 5 μg tRNA$^{[Ser]Sec}$ DNA; lane 3, G21/D10 + 20 μg tRNA$^{[Ser]Sec}$ DNA; lane 4, D10 vector control; lane 5, RM2/D10. Western analysis was performed with peptide 1 antisera. The positions of full-length and UGA-terminated proteins are indicated by arrows.

Table III. Effects of tRNA$^{[Ser]Sec}$ anticodon mutants on readthrough of type I deiodinase Sec codon mutants

Codon 126	tRNA$^{[Ser]Sec}$ anticodon	tRNA DNA (μg)	Relative activity (c.p.m. I^-/hGH)
UGA		0	168
UAA		0	1.2
UAA	UUA	2	2.9
UAA	UUA	10	10.5
UAA, del SECIS	UUA	0	0.4
UAA, del SECIS	UUA	10	0.5
UUA		0	0
UUA	UAA	2	0.9
UUA	UAA	10	3.4

DNA concentrations are given as μg plasmid transfected/60 mm plate of 293 cells. Deiodinase activity is expressed as c.p.m. I^- released/μl protein, normalized to c.p.m. hGH released in the media. Transfections contained 5 μg mutant deiodinase plasmid, 5 μg plasmid D15, and indicated amounts of tRNA-expressing plasmids.

the amount of 28 kDa protein (Fig. 4). This assay measures the suppression of UGA, which is a different process than Sec incorporation, as it does not require either a specialized elongation factor or a SECIS element. Clearly, the suppressor tRNA must compete with the release factor for UGA. The fact that such low readthrough of UGA occurs in lysates programmed with G21 RNA, but yet supplementing reaction mixtures with tRNA$^{[Ser]Sec}$ enhances readthrough, demonstrates that the endogenous level of tRNA$^{[Ser]Sec}$ is insufficient in lysates to adequately compete with the release factor.

Complementary mutations of the selenocysteine codon and tRNA anticodon allow readthrough

To determine if the identity of UGA is critical for Sec incorporation into selenoproteins in mammalian cells, we utilized two deiodinase mutants in which UGA had been changed to either a UAA termination codon or a UUA leucine codon. We had previously demonstrated that the UAA mutant produces only the

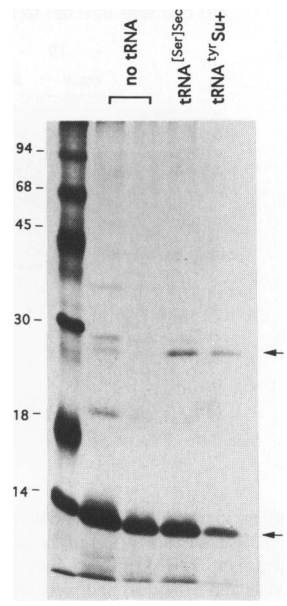

Figure 4. Effects of tRNA$^{[Ser]Sec}$ and tRNA^{Tyr} suppressor on *in vitro* translation of 5′ deiodinase. *In vitro* translation reaction mixtures were immunoprecipitated with peptide 1 antisera. Lane 1, MW markers; lanes 2 and 3, G21 RNA; lane 4, G21 RNA + bovine liver tRNA$^{[Ser]Sec}$; lane 5, G21 RNA + yeast tRNA^{Tyr}Su+. The positions of full-length and UGA-terminated proteins are indicated by arrows.

truncated protein, and no deiodinase activity, while the UUA leucine mutant produces a full-length protein which is enzymatically inactive (6). The anticodon of the *Xenopus* tRNA$^{[Ser]Sec}$ gene was mutated to generate complementarity to the above two codon mutants, and these tRNAs were cotransfected with their corresponding mutant deiodinase constructs. The results in Table III show that the identity of the Sec codon as UGA is not required for Sec incorporation, as long as two requirements are met; codon–anticodon complementarity, and the presence of a SECIS element. Translation of the UAA mutant to produce a functional enzyme occurs in a tRNA$^{[Ser]Sec}$UUA dose-dependent manner, but is abolished by deletion of the SECIS element. However, the levels of deiodinase activity even with the highest amount of tRNA tested were 10- to 30-fold lower than that produced by G21 without added tRNA. The UUA leucine mutant and corresponding tRNA also produce a functional enzyme, but the activity levels were even lower, indicating that Sec-tRNA$^{[Ser]Sec}$UAA is able to compete with leucyl-tRNA^{Leu}UAA, albeit at a very low efficiency.

Effects of selenium on UGA readthrough

The availability of selenium in the media has been reported to have varying effects on selenoprotein expression in different mammalian cells (see 1 for review and 22 for subsequent work). That is, the regulatory role of selenium in selenoprotein expression has been attributed to effects at the level of transcription, mRNA stability or translation depending on the selenoprotein and cell type. We examined the effects of selenium availability on translation of the deiodinase in transiently transfected cells (see Fig. 5 and Table IV). The amount of full-length protein was severely diminished in cells cultured in 1% serum containing trace amounts of selenite as compared to cells grown under the same conditions except that the media was

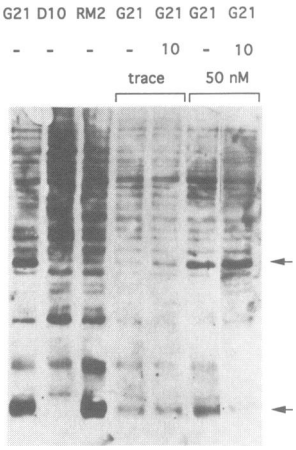

Figure 5. Effects of selenium in media on UGA codon readthrough. Transfections in lanes 1–3 contained 10% fetal calf serum in the media. Lane 1, G21/D10; lane 2, D10 vector control; lane 3, RM2/D10. Lanes 4–7 contained 1% serum + hormones (see Materials and Methods) and were transfected with G21/D10. Lanes 5 and 7 also received 10 μg *Xenopus* $tRNA^{[Ser]Sec}$ DNA. Lanes 6 and 7 were supplemented with 50 nM selenium at the time of transfection. Lanes 4 and 5 received no Se. Western analysis was performed with peptide 1 antisera. Lane 7 was taken from a different exposure from the rest of the gel, due to an opacity in the film in this region. The positions of full-length and UGA-terminated proteins are indicated by arrows.

Table IV. Effects of selenium and $tRNA^{[Ser]Sec}$ on type I deiodinase activity

Se (nM)	$tRNA^{[Ser]Sec}$ DNA (μg)	Relative activity (c.p.m. I^-/hGH)
~0.2	0	7.6
~0.2	10	5.9
50	0	286
50	10	397

Deiodinase activity is expressed as c.p.m. I^- released/μl protein, normalized to c.p.m. hGH released in the media. All transfections contained 5 μg plasmid D15. Other conditions are given in Materials and Methods.

supplemented with 50 nM selenite (see lanes 4 and 6 in Fig. 5). The UGA-terminated 14 kDa protein, on the other hand, was only slightly decreased by selenium depletion as shown in lanes 4 and 6. Addition of 10 μg $tRNA^{[Ser]Sec}$ to the transfections increased the amount of full-length protein, while significantly diminishing the amount of truncated protein in the selenium sufficient cells (compare lanes 6 and 7). Inclusion of $tRNA^{[Ser]Sec}$ in transfections with selenium depleted cells had only a slight effect on the levels of the 14 and 28 kDa proteins (compare lanes 4 and 5). Lanes 1–3 in Fig. 5 show the results of transfecting the cells with the G21 (wild-type), D10 (vector control), and RM2 (stem–loop mutant). These studies were included as controls and the data are similar to those found in other studies (e.g. see Figures 2 and 3).

The deiodinase activities were also measured in these cells (see Table IV) and the results are consistent with those shown in Figure 5. The deiodinase activity decreased 30- to 60-fold compared to cells cultured in the presence of 50 nM selenite. Transfer $RNA^{[Ser]Sec}$ had little or no effect on deiodinase activity in selenium depleted cells, while the activity was increased about 1.4-fold in selenium sufficient cells in response to $tRNA^{[Ser]Sec}$.

DISCUSSION

In a previous study (7), we examined the relative efficiencies of expression of a wild-type selenoprotein and its cysteine mutant analog. The study used transient transfection to express the two proteins and showed that this system is competent for normal (cysteine mutant) protein synthesis, but that a defect existed in the selenoprotein synthesis pathway. Although transfection elevates the concentrations of the mRNAs for the two proteins well above the normal *in vivo* level, the endogenous levels of ribosomes, tRNAs, and elongation factors are sufficient to translate the cysteine mutant protein at 20- to 400-fold higher levels than the selenocysteine-containing protein. Since the only difference between the two transfected cDNAs was the substitution of a cysteine codon for a selenocysteine codon, this indicates a specific deficiency in one or more of the selenocysteine specific factors, e.g. $tRNA^{[Ser]Sec}$, selenocysteine synthase, selenium donor protein, and/or the putative selenocysteine-specific elongation factor. The nature of this deficiency was not previously identified; therefore, the current study was undertaken to address this question.

We found that by decreasing the amount of deiodinase cDNA transfected, we could increase the efficiency of selenocysteine codon readthrough, suggesting limiting amounts of a required factor. Experiments with cotransfected $tRNA^{[Ser]Sec}$ show that it is, in fact, at least one of the limiting components. However, the increase in activity with cotransfected wild-type tRNA was less than two-fold, and the change in ratio of full-length to truncated protein was only six-fold. In contrast, the difference between cysteine mutant and wild-type protein expression levels in the 293 cell line is ~100-fold (data not shown). Thus, addition of the tRNA only partially overcomes the defect, indicating that it is one, but not the only limiting factor. This result demonstrates that both the tRNA and other factors specific for selenoprotein synthesis in mammalian cells limit the efficiency of UGA readthrough, as compared to cysteine codon translation. The nature and availability of the other factors involved in selenoprotein synthesis must await their isolation or reagents for their detection and quantitation.

We next examined the ability of complementary mutations of the selenocysteine codon and the tRNA anticodon to allow readthrough of the codon. Translation of the UAA mutant deiodinase construct does not appear to occur in the absence of added mutant tRNA even though misreading of certain UAA codons by normal cellular tRNAs has been reported in mammalian cells (23). Thus, normal tRNAs apparently do not compete with release factor for recognition of this UAA. Competition between $tRNA^{[Ser]Sec}$ and release factor obviously occurs to some extent in translation of the UGA codon in the wild-type enzyme, and we show here that it also occurs with the UAA mutant in the presence of cotransfected mutant $tRNA^{[Ser]Sec}UUA$.

In contrast, the UUA leucine codon is translated efficiently by the normal leucyl-$tRNA^{Leu}UAA$. Thus, incorporation of Sec in place of leucine to produce a functional enzyme would require competition between the putative eukaryotic SELB homolog–Sec-$tRNA^{[Ser]Sec}UAA$ complex and normal translation of the UUA codon by elongation factor EF-1-bearing leucyl-$tRNA^{Leu}UAA$. We find that $tRNA^{[Ser]Sec}UAA$ is able to compete for the codon, albeit inefficiently. This indicates that the ability of the putative eukaryotic SELB homolog, in conjunction with a SECIS element, to direct Sec incorporation is efficient enough

to compete, at least to some extent, with both release factor-mediated termination and EF-1-mediated translation. It should be noted that Bock and collaborators have also found that complementarity between mutant Sec codons and the corresponding Sec-tRNA anticodon support insertion of this amino acid into protein in prokaryotes (24).

Nonetheless, Sec insertion is much less efficient with either mutant than in the wild-type deiodinase. Affinity of release factor is reportedly higher for UAA than for UGA (25) but not enough to account for the differences observed here. Thus the explanation for the reduced efficiency in the case of UAA is not clear.

A question has arisen as to whether there is a specific mechanism preventing release factor-mediated termination at Sec codons. Bock *et al.* suggested that the stem−loop in fdH might block access of RF-2 to the UGA codon (26). Thus, it is not known whether competition between the release factor and the translation machinery plays an important regulatory role in selenoprotein expression. The results with the deiodinase stem−loop mutant suggest that in the absence of a functional SECIS element, this UGA is, in fact, able to function as a stop codon. If the ribosomes were paused at this position and did not terminate, each mRNA would produce at most one paused product, which may be released upon disruption of cells, but the mRNAs would not be available for multiple rounds of translation. The high levels of truncated product produced by the mutant argue against this. Even in the presence of a functional SECIS element, particularly when mRNA levels are high, competition between termination and readthrough may regulate levels of the full-length active protein. This competition would also come into play under conditions of limiting selenium, as evidenced by the selenium deprivation study reported herein.

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