



Processive Selenocysteine Incorporation during Synthesis of Eukaryotic Selenoproteins

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Selenoproteins are a family of proteins that share the common feature of containing selenocysteine, the “twenty-first” amino acid. Selenocysteine incorporation occurs during translation of selenoprotein messages by redefinition of UGA codons, which normally specify termination of translation. Studies of the eukaryotic selenocysteine incorporation mechanism suggest that selenocysteine insertion is inefficient compared with termination. Nevertheless, selenoprotein P and several other selenoproteins are known to contain multiple selenocysteines. The production of full-length (FL) protein from these messages would seem to demand highly efficient selenocysteine incorporation due to the compounding effect of termination at each UGA codon. We present data demonstrating that efficient incorporation of multiple selenocysteines can be reconstituted in rabbit reticulocyte lysate translation reactions. Selenocysteine incorporation at the first UGA codon is inefficient but increases by approximately 10-fold at subsequent downstream UGA codons. We found that ribosomes in the “processive” phase of selenocysteine incorporation (i.e., after decoding the first UGA codon as selenocysteine) are fully competent to terminate translation at UAG and UAA codons, that ribosomes become less efficient at selenocysteine incorporation as the distance between UGA codons is increased, and that efficient selenocysteine incorporation is not dependent on *cis*-acting elements unique to selenoprotein P. Furthermore, we found that the percentage of ribosomes decoding a UGA codon as selenocysteine rather than termination can be increased by 3- to 5-fold by placing the murine leukemia virus UAG read-through element upstream of the first UGA codon or by providing a competing messenger RNA *in trans*. The mechanisms of selenocysteine incorporation and selenoprotein synthesis are discussed in light of these results.

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Introduction

Selenium has long been known for its beneficial antioxidant properties, which are attributable to incorporation of this trace element into selenopro-

teins. Incorporation of selenium in the form of selenocysteine, the twenty-first amino acid, occurs co-translationally and is encoded in a subset of messages by UGA codons. The greater effectiveness of selenocysteine than cysteine for catalyzing redox reactions is likely the reason nature has evolved and maintained the specialized machinery required to produce and incorporate selenocysteine into proteins.

Ribosome reprogramming to decode a UGA codon as selenocysteine requires *cis*-acting signals in the RNA and a number of *trans*-acting factors. In eukaryotes, a secondary structure called the selenocysteine insertion sequence (SECIS) element is found in the 3' untranslated region (3' UTR) of

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Abbreviations used: FL, full length; SECIS, selenocysteine insertion sequence; SRE, selenocysteine redefinition element; SBP2, SECIS binding protein 2; UTR, untranslated region; SelN, selenoprotein N; SelT, selenoprotein T; SelP, selenoprotein P; MuLV, murine leukemia virus; RRL, rabbit reticulocyte lysate.

selenoprotein messages. The presence of the SECIS element is necessary to direct message-specific selenocysteine incorporation at UGA codons.¹⁻³ Another *cis*-acting element that increases the efficiency of selenocysteine incorporation is a selenocysteine redefinition element (SRE) found immediately downstream of UGA codons in a subset of selenoprotein messages.^{4,5} SECIS binding protein 2 (SBP2), a *trans*-acting factor, has been shown to bind to eukaryotic SECIS elements^{6,7} and the ribosome.^{7,8} SBP2 recruits EFsec,^{9,10} a specialized elongation factor^{10,11} that ultimately delivers the Sec-tRNA^{[Ser]Sec} to the decoding center of the ribosome. Other *trans*-acting factors have recently been implicated in the selenocysteine incorporation mechanism and its regulation, including the ribosomal protein L30,¹² nucleolin,^{13,14} and eIF4a3,¹⁵ each of which can interact directly with SECIS elements. However, the exact roles of these latter proteins are not yet clear.

Of direct importance to the production and regulation of eukaryotic selenoproteins is the efficiency of selenocysteine incorporation. For selenoprotein genes with a single selenocysteine-encoding UGA codon, termination appears to be the predominant event in *Escherichia coli*,¹⁶ in rabbit reticulocyte *in vitro* translation reactions,^{17,18} and in transiently transfected mammalian cells.^{10,19} This suggests that selenocysteine incorporation is inherently inefficient relative to termination.¹⁹

Several selenoprotein genes have now been identified with the potential to encode more than one selenocysteine residue. These include iodothyronine deiodinase 2, an alternatively spliced isoform of selenoprotein N (SelN alternate transcript 1), selenoprotein L, and selenoprotein P (SelP). Iodothyronine deiodinase 2, SelN transcript 1, and selenoprotein L have two UGA codons each. The total number of selenocysteines in SelP ranges among species from 10 in humans to as many as 28 in sea urchins.²⁰ The second and all downstream selenocysteine amino acids cluster near the C-terminus of SelP and appear to be required in mammals for delivery of selenium from the liver to various other tissues, most importantly the brain and testes.²¹ Purification of SelP from rat plasma revealed several isoforms of the protein. These isoforms were shown by carboxypeptidase sequencing²² and mass spectrometry²³ to comprise a significant amount of full-length (FL) protein and several peptides that were prematurely UGA terminated. In contrast to the model of inefficient selenocysteine incorporation described above, production of FL SelP would require highly efficient selenocysteine incorporation due to the cumulative loss of product caused by termination at each UGA codon.

It has been suggested that SelP may utilize a special mechanism for selenocysteine incorporation due to the exceptional number of UGA codons and the observation that SelP messages are unique in having two conserved 3' UTR SECIS elements. A recent study²⁴ suggested that the two SECIS

elements in the zebrafish SelP message have different functions. In this model, the second SECIS, SECIS2, serves to incorporate selenocysteine at the first UGA. It does so inefficiently, acting as a checkpoint for selenocysteine incorporation factors and to restrict the number of downstream ribosomes. The first SECIS, SECIS1, is then dedicated for redefinition of the remaining 16 UGA codons near the 3' end of the open-reading frame. It was concluded that the SelP gene has evolved unique properties to accommodate the incorporation of multiple selenocysteines into one polypeptide.

The results presented here demonstrate that efficient selenocysteine incorporation can be reconstituted in rabbit reticulocyte lysate (RRL) translation reactions. As previously reported for SelP sequences expressed in cultured cells,²⁴ selenocysteine incorporation was inefficient at the first UGA but highly processive at downstream UGA codons. We demonstrate that processivity of selenocysteine incorporation is not dependent on sequence elements unique to SelP but can be recapitulated with SECIS elements from other selenoprotein messages or when the UGA codons are in non-SelP sequence contexts. A revised model for selenocysteine incorporation is proposed based on the results presented below.

Results

The efficiency of UGA codon redefinition to encode selenocysteine was measured using a dual-luciferase reporter vector, pDluc, designed for expression of the reporter genes in RRL transcription and translation reactions or in cultured cells. The pDluc constructs used here contain both the Renilla and firefly luciferase reporter genes flanking the UGA codon(s) of interest and a SECIS element inserted downstream of the firefly luciferase gene in the 3' UTR to support selenocysteine insertion (Materials and Methods). The following constructs were transcribed and translated in RRL supplemented with the 447 C-terminal amino acids of SBP2 (SBP2-CT), which have been shown to be proficient for selenocysteine incorporation.⁷ It is necessary to separate the resulting radiolabeled protein products by SDS-PAGE (polyacrylamide gel electrophoresis) to determine the efficiency of selenocysteine insertion at each position during translation of messages containing more than one UGA codon. The products corresponding to termination at each UGA, UAA, or UAG codon were quantified by phosphorimaging analysis of SDS-PAGE gels, and the efficiency of selenocysteine incorporation or ribosome read-through was determined by comparison of the quantified products. Where there is more than one UGA codon, the reported efficiency of selenocysteine incorporation at downstream UGA codons takes into account only those ribosomes that have read through the upstream UGA codons (Materials and Methods). Verification that read-through of a UGA

codon was due to selenocysteine incorporation and not near-cognate tRNA decoding at the UGA codon was provided by the dependence of read-through on a SECIS element in the 3' UTR, the UGA codon, and the addition of SBP2-CT to the translation reaction. No read-through of UGA codons was observed when the SECIS element was lacking, the UGA codon was changed to UAG or UAA, or SBP2-CT was excluded from the reaction.

Selenocysteine incorporation efficiency during translation of messages containing more than one UGA codon

To determine the efficiency of selenocysteine incorporation when two UGA codons were present in one messenger RNA (mRNA), we created a reporter construct P2/3 in which 130 nt containing the second and third UGA codons of the human SelP gene were cloned between the Renilla and firefly genes of pDluc containing SECIS1 and SECIS2 of SelP downstream of the firefly coding sequence (Fig. 1a) (see Materials and Methods, S1/S2). To determine if read-through at the second UGA codon was due to a general decrease in termination efficiency, we made two additional reporter constructs in which the second UGA codon was changed to either UAA or UAG—P2/3UAA or P2/3UAG, respectively.

These constructs were transcribed and translated in SBP2-CT-supplemented RRL reactions. The resulting L-[³⁵S]methionine-labeled products were separated by SDS-PAGE (Fig. 1b), and the termination and FL products were quantified as described in Materials and Methods. Read-through of the first UGA was inefficient with approximately 7% selenocysteine incorporation [Fig. 1b, lane 6 (term 1), and Fig. 1c]. Read-through of the second UGA codon occurred with much higher efficiency, 67% ($p < 0.001$), a nearly 10-fold increase [Fig. 1b, lane 6 (term 2), and Fig. 1c]. No FL product was detectable when the second UGA was changed to UAA or UAG (Fig. 1b, lanes 2 and 4) or when SBP2-CT was excluded from the RRL transcription and translation reactions (Fig. 1b, lanes 3, 5, and 7).

SECIS effects on selenocysteine incorporation efficiency

To determine the requirement for the two SelP SECIS elements to support high-level decoding of the second UGA as selenocysteine, we replaced SECIS1 and SECIS2 (S1/S2) with the first SECIS element alone (P2/3S1), the second SECIS element alone (P2/3S2), the SECIS element from SelN (P2/3SN), or the SECIS element from selenoprotein T (SelT) (P2/3ST) (Fig. 2a). Selenocysteine insertion at the first UGA codon was inefficient, ranging from 2% to 7% depending on the identity of the SECIS element, and in all cases, selenocysteine insertion was significantly higher at the second UGA codon: P2/3S1, 6% and 63%; P2/3[S1/S2],

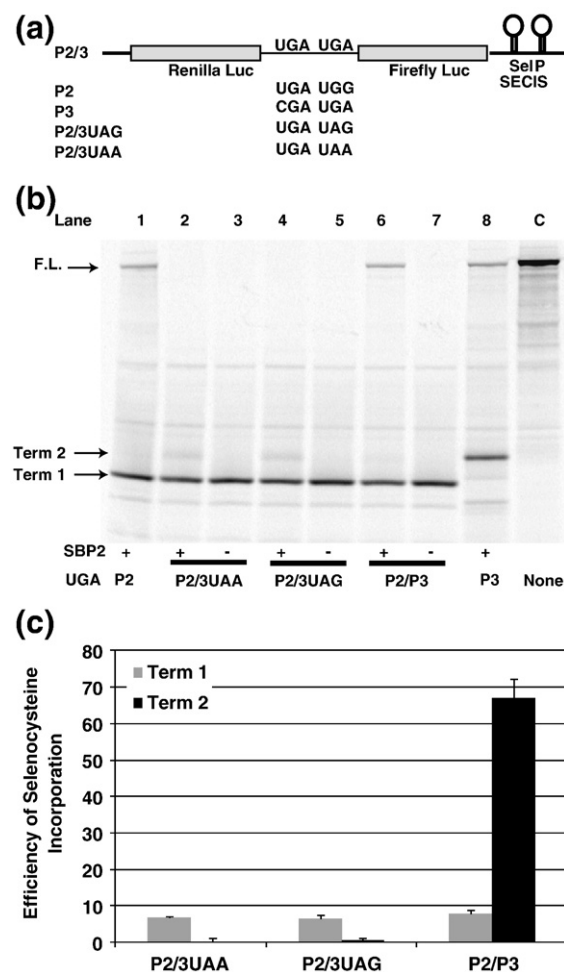


Fig. 1. Selenocysteine incorporation efficiency during decoding of a message with two UGA codons. (a) Schematic representation of the reporter messages: P2/3 is shown in whole. P2, P3, P2/3UAG, and P2/3UAA are identical, except for the indicated changes to the UGA codons. (b) ³⁵S-labeled proteins from RRL transcription and translation reactions in the presence (+) or absence (–) of SBP2-CT (SBP2) were separated by SDS-PAGE. The messages translated were as follows: lane 1, P2; lanes 2 and 3, P2/3UAA; lanes 3 and 4, P2/3UAG; lanes 5 and 6, P2/3; lane 6, P3; lane 7, control with no UGA codons. The positions for protein products corresponding to termination at the first (Term 1) or second (Term 2) UGA/UAG/UAA and the FL protein are indicated by arrows. (c) Quantitative analysis of selenocysteine incorporation efficiency at the first and second selenocysteine/termination codons for P2/3UAA, P2/3UAG, and P2/3. Bars are the average of two independent experiments ($n=6$). Error bars are the standard deviation from the mean.

7% and 57%; P2/3SN, 4% and 36%; P2/3ST, 4% and 41%; P2/3S2, 2% and 12% (Fig. 2b, lanes 2, 4, 6, 8, and 10, and Fig. 2c) ($p < 0.001$). Consistent with previously published results,²⁴ selenocysteine insertion efficiency for SECIS1 from SelP was not significantly different from that observed with the combination of SECIS1 and SECIS2. It is notable that there was a strong positive correlation between the efficiency of read-through of the

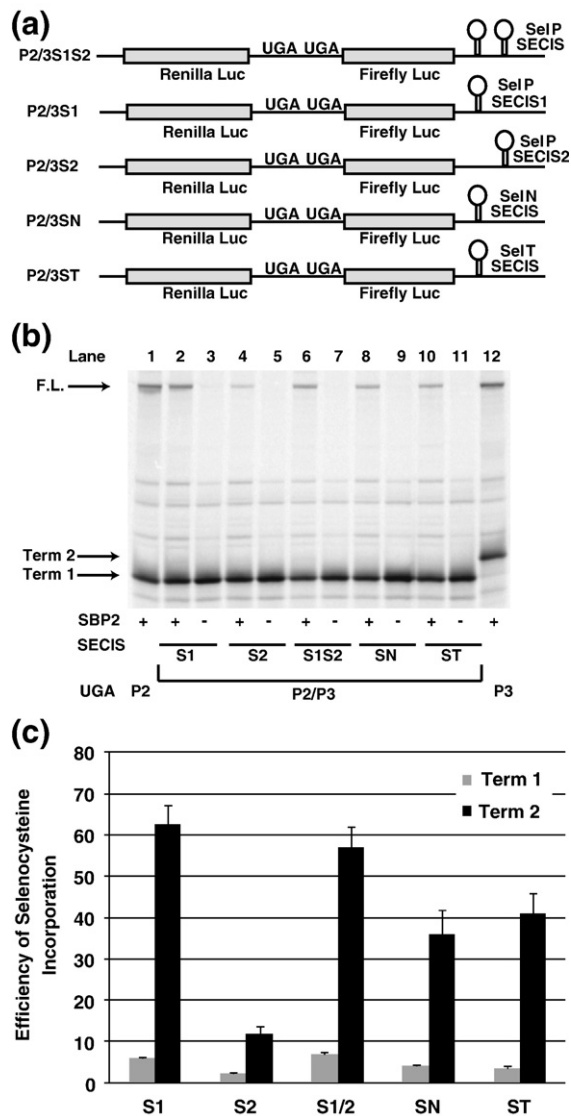


Fig. 2. SECIS effects on selenocysteine incorporation efficiency during decoding of a message with two UGA codons. (a) Schematic representation of the reporter messages: P2/3 with the SECIS elements from SelP (2/3S1S2, P2/3S1, P2/3S2), SelN (P2/3SN), and SelT (P2/3ST) cloned into the 3' UTR. (b) ^{35}S -labeled proteins resulting from RRL transcription and translation reactions in the presence (+) or absence (-) of SBP2-CT (SBP2) were separated by SDS-PAGE. The messages translated were as follows: lane 1, P2; lanes 2 and 3, P2/3S1; lanes 4 and 5, P2/3S2; lanes 6 and 7, P2/3S1S2; lanes 8 and 9, P2/3SN; lanes 10 and 11, P2/3ST; lane 12, P3. The positions for protein products corresponding to termination at the first (Term 1) or second (Term 2) UGA and the FL protein are indicated by arrows. (c) Quantitative analysis of selenocysteine incorporation efficiency at the first and second selenocysteine/termination codons. Bars are the average of two independent experiments ($n=6$). Error bars are the standard deviation from the mean.

second UGA and that of the first UGA (see Discussion; Fig. 8), which was dependent upon the identity of the SECIS element.

Distance effects on selenocysteine incorporation efficiency

With the exception of the first selenocysteine-encoding UGA codon in SelP, all downstream UGA codons are clustered near the 3' end of the SelP message. To determine if the proximity of the downstream UGA codon relative to the upstream UGA codon could influence the efficiency of selenocysteine incorporation, we increased the distance between the two UGA codons in P2/3. The distance between the two UGA codons in P2/3 is 51 nt, as it is in the wild-type human SelP message. This distance was increased by insertion of random sequences to 78 and 108 nt to create the reporter constructs P2/3_78, and P2/3_108, respectively (Fig. 3a). A small decrease in selenocysteine incorporation efficiency was observed when the distance between UGA codons was increased from 51 nt (78%) to 108 nt (66%) ($p<0.01$) (Fig. 3b, lanes 1, 3, and 5, and Fig. 3c).

To examine the effect of greater distances between the first and second UGA codons, we cloned sequences from the beginning of SelP through SelP UGA 10 into pDlucS1/S2. UGA 3–UGA 9 were changed to sense codons (Fig. 4a; see Materials and Methods). In messages produced from the reporter construct P726, the distance between the first and second UGA codons is 726 nt. This distance was reduced by making sequential deletions to 516 nt (P516), 312 nt (P312), and 96 nt (P96). The efficiency of selenocysteine incorporation at the first and second UGA codons was determined (Fig. 4b) as described above, with the exception that termination at the third UGA (SelP UGA 10) was excluded from the calculation because no band could be detected. Selenocysteine incorporation rates during decoding of the second UGA codon were determined to be 32% for P726, 37% for P516, 45% for P312, and 50% for P96 (Fig. 4b, lanes 1, 2, 3, and 4, and Fig. 4c). Increasing the distance between the first and second UGA codons by 630 nt reduced selenocysteine incorporation by nearly 2-fold from 50% to 32% ($p<0.01$). Selenocysteine incorporation at the first UGA codon was approximately 5% in all cases and unaffected by the distance between the first and second UGA codons.

Sequence context effects on selenocysteine incorporation efficiency

In the experiments described above, all UGA codons and their surrounding sequence contexts were derived from the human SelP gene, which has been postulated to have special signals or sequence contexts that contribute to highly efficient selenocysteine incorporation.²⁴ To examine the effect of sequence context on selenocysteine incorporation efficiency, we cloned three reporter vectors with one (1UGA/UAA), two (2UGA), or four (4UGA) selenocysteine codons in a random

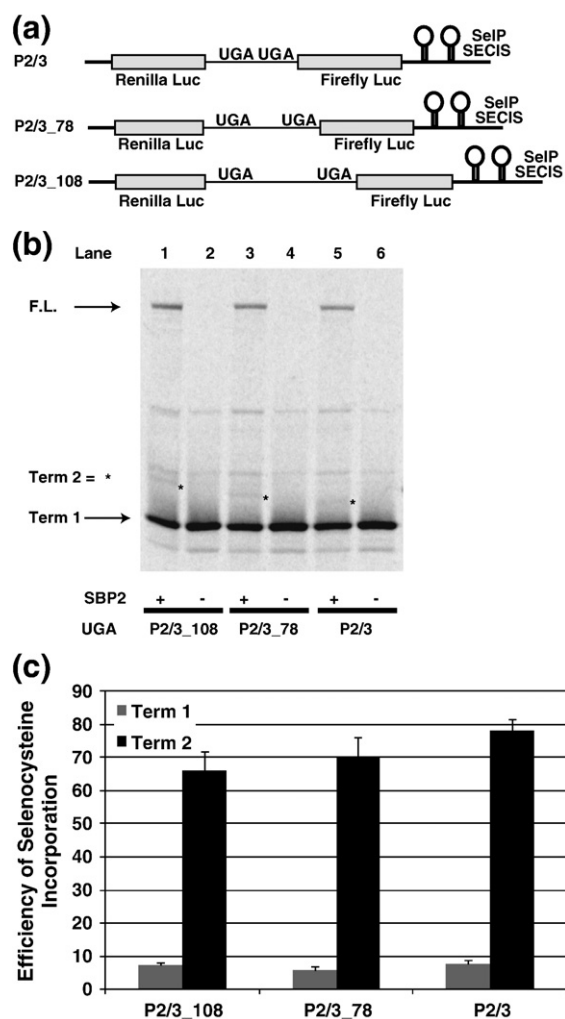


Fig. 3. Distance effects on selenocysteine incorporation efficiency during decoding of a message with two UGA codons. (a) Schematic representation of the reporter messages: P2/3, and with sequence insertions between the two UGA codons P2/3_78 and P2/3_108. (b) ³⁵S-labeled proteins resulting from RRL transcription and translation reactions in the presence (+) or absence (-) of SBP2-CT (SBP2) were separated by SDS-PAGE. The messages translated were as follows: lanes 1 and 2, P2/3_108; lanes 3 and 4, P2/3_78; lanes 5 and 6, P2/3. The positions for protein products corresponding to termination at the first UGA (Term 1) and the FL protein are indicated by arrows. The positions for protein products arising from termination at the second UGA (Term 2) are indicated by asterisks. (c) Quantitative analysis of selenocysteine incorporation efficiency at the first and second UGA codons. Bars are the average of two independent experiments ($n=6$). Error bars are the standard deviation from the mean.

sequence into pDlucS1S2 between the Renilla and firefly reporter genes (Fig. 5a). These constructs were transcribed and translated, and the radiolabeled products were analyzed by SDS-PAGE (Fig. 5b). As seen above, selenocysteine incorporation efficiency at the first UGA codon was inefficient (~10%) and highly efficient (between

68% and 87%) at all subsequent downstream UGA codons (Fig. 5b, lanes 1, 3, and 5, and Fig. 5c). This finding demonstrates that although sequence context does have an influence on absolute levels of selenocysteine incorporation, special sequence contexts are not required for increased efficiency of selenocysteine incorporation following decoding at the first UGA codon.

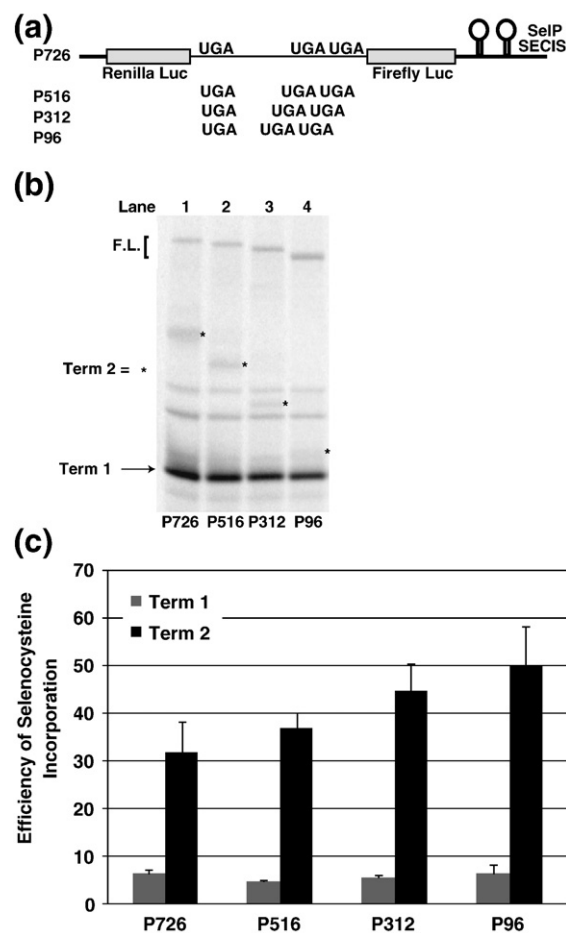


Fig. 4. Distance effects on selenocysteine incorporation efficiency during decoding of a message with three UGA codons. (a) Schematic representation of the reporter messages: P726, and with sequential deletions between the first two UGA codons P516 and P312, P96. (b) ³⁵S-labeled proteins resulting from RRL transcription and translation reactions supplemented with SBP2-CT were separated by SDS-PAGE. The messages translated were as follows: lane 1, P726; lane 2, P516; lane 3, P312; lane 4, P96. The positions for protein products corresponding to termination at the first UGA (Term 1) and the FL protein are indicated by an arrow and bracket, respectively. The positions for protein products arising from termination at the second UGA (Term 2) are indicated by asterisks. (c) Quantitative analysis of selenocysteine incorporation efficiency at the first and second UGA codons for P726, P516, P312, and P96. Bars are the average of two independent experiments ($n=6$). Error bars are the standard deviation from the mean.

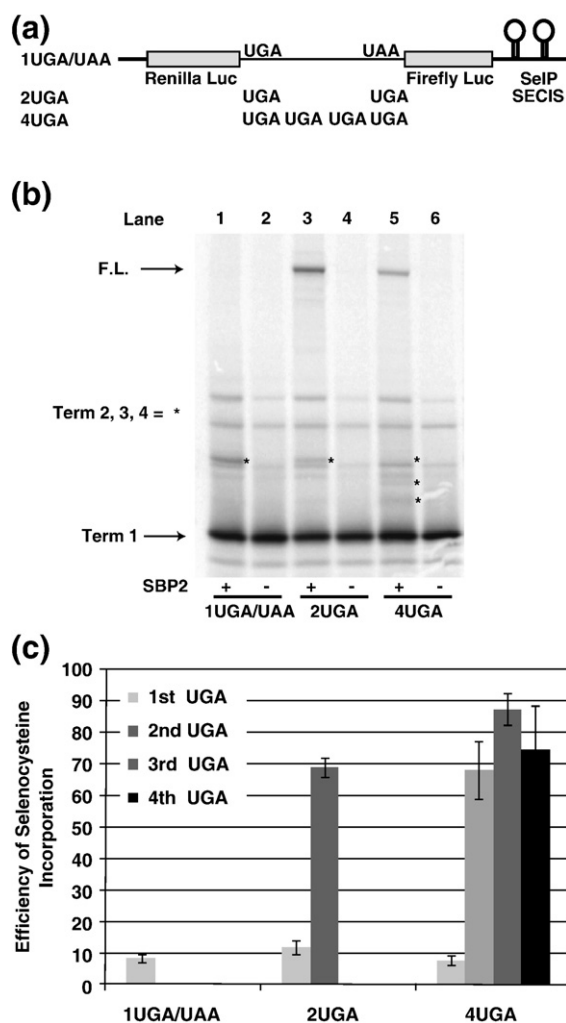


Fig. 5. Local sequence context effects on selenocysteine incorporation efficiency during decoding of a message with multiple UGA codons. (a) Schematic representation of the reporter messages: 1UGA/UAA contains a UGA codon and a UAA codon in a random sequence context; 2UGA and 4UGA contain two and four UGA codons, respectively, embedded in the same sequence context. (b) ^{35}S -labeled proteins resulting from RRL transcription and translation reactions supplemented with (+) or without (-) SBP2-CT (SBP2) were separated by SDS-PAGE. The messages translated were as follows: lanes 1 and 2, 1UGA/UAA; lanes 3 and 4, 2UGA; lanes 5 and 6, 4UGA. The positions for protein products corresponding to termination at the first UGA and the FL protein are indicated by arrows. The positions for protein products arising from termination at the second, third, or fourth UGA (or UAA) codons are indicated by asterisks. (c) Quantitative analysis of selenocysteine incorporation efficiency at the UGA codons for each construct. Bars are the average of two independent experiments ($n=6$). Error bars are the standard deviation from the mean.

Read-through of the MuLV UAG redefinition element or addition of a competing mRNA can increase selenocysteine incorporation efficiency at UGA codons

The results presented above demonstrate that, under a variety of conditions, the first UGA is

decoded as selenocysteine with low efficiency and all subsequent UGA codons are read through with much higher efficiency. In order to determine if this effect is dependent upon selenocysteine insertion at the first UGA, we replaced the first UGA in P2/3 with approximately 80 nt from the murine leukemia virus (MuLV read-through cassette) that includes a UAG codon and a pseudo-knot structure that induces read-through of the UAG codon (MuLV/P3; Fig. 6a). The MuLV relies on read-through of this UAG codon to express the gag-pol polyprotein. This case of stop-codon redefinition is selenocysteine independent, and it has been shown that the UAG is decoded as glutamine²⁵ with approximately 5% efficiency.^{26,27} In good agreement with this value, we observed approximately 6% read-through of the UAG codon (Fig. 6b, lane 1, and Fig. 6c), which was unaffected by the presence of SBP2-CT (Fig. 6b, lane 2). Read-through of the UAG codon was sufficient to elevate selenocysteine incorporation during decoding of the downstream UGA codon to approximately 30%, 3-fold higher than the 10% ($p<0.001$) selenocysteine incorporation efficiency observed when the UAG was changed to a CAG or P2 was changed to a sense codon (MuLV/CAG/P3 and P3). To determine if read-through of the MuLV UAG codon results in a general increase in stop-codon redefinition efficiency, we tested a dual-MuLV construct in which two MuLV read-through cassettes were inserted in tandem into pDlucS1S2 (MuLV/MuLV; Fig. 6a). As with the MuLV/P3 construct, read-through efficiency of the second UAG codon increased by nearly 2-fold ($p<0.01$) (Fig. 6b, lane 7, and Fig. 6c). Read-through of the UAG codons was shown to be unaffected by the addition of SBP2-CT (data not shown).

Inclusion of the MuLV read-through cassette in a message reduces the number of ribosomes translating downstream due to release of ribosomes that terminate translation. Constructs containing either a single UGA codon (P3) or a single MuLV read-through cassette (MuLV) (Fig. 7a) were transcribed and translated in the presence of increasing amounts of a competing mRNA (Fig. 7b; Materials and Methods) to determine if a general reduction in translation was sufficient to increase read-through efficiency. As expected, increasing amounts of competitor resulted in decreased product produced from both P3 and MuLV messages (Fig. 7b, compare lanes 1–4 and 5–8, respectively). However, read-through efficiency increased by up to 5-fold for both selenocysteine incorporation and UAG redefinition at the highest level of competitor tested ($p<0.001$) (Fig. 7c). These results demonstrate that inefficient decoding of a UAG codon or reduced translation of messages due to competition with an mRNA provided *in trans* is sufficient to increase the efficiency of selenocysteine incorporation at downstream UGA codons.

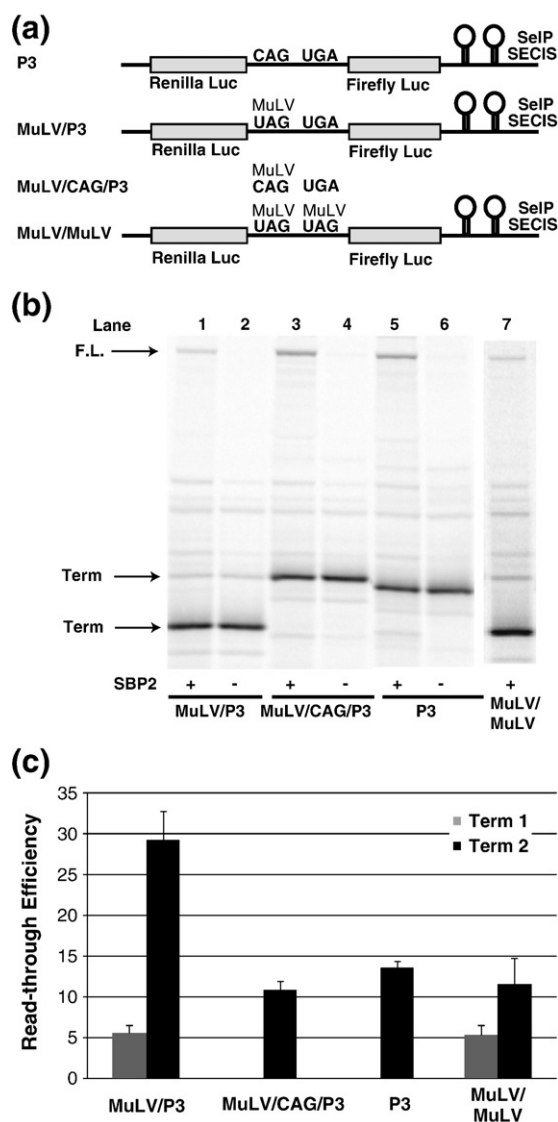


Fig. 6. Read-through of the MuLV UAG codon increases selenocysteine incorporation at a downstream UGA codon and read-through of a second downstream MuLV UAG codon. (a) Schematic representation of the reporter messages: P3 contains a UGA codon, MuLV/P3 has the MuLV UAG codon and pseudoknot stimulator of read-through inserted upstream of the UGA codon, MuLV/CAG/P3 is the same as MuLV/P3 except that the UAG is mutated to a CAG, and MuLV/MuLV has two MuLV read-through cassettes in tandem. (b) ^{35}S -labeled proteins resulting from RRL transcription and translation reactions supplemented with (+) or without (-) SBP2-CT were separated by SDS-PAGE. The messages translated were as follows: lanes 1 and 2, MuLV/P3; lanes 3 and 4, MuLV/CAG/P3; lanes 5 and 6, P3; lane 7, MuLV/MuLV. The positions for protein products arising from termination (Term) at the UAG codon, the UGA codon, and the FL protein are indicated by arrows. (c) Quantitative analysis of selenocysteine incorporation or read-through efficiency at the UGA or UAG codons for each construct. Bars are the average of two independent experiments ($n=6$). Error bars are the standard deviation from the mean.

Discussion

While significant advances have been made in our understanding of the selenocysteine insertion mechanism and its role in selenoprotein synthesis in eukaryotes, fundamental questions remain to be answered. The discovery of the SelP gene containing multiple UGA codons poses a conceptual challenge to the model of non-processive selenocysteine incorporation. How can two SECIS elements reprogram ribosomes to insert selenocysteine at multiple

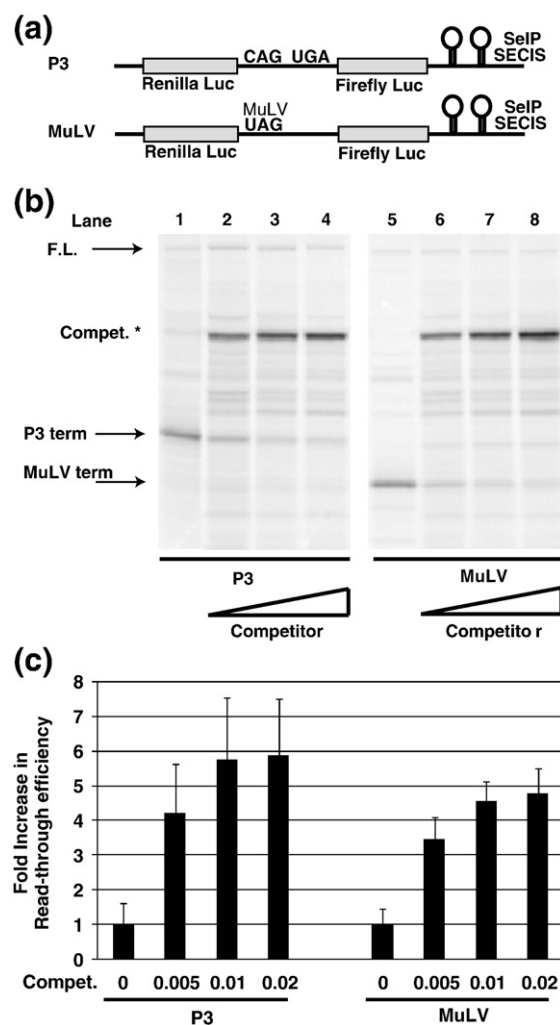


Fig. 7. Competing mRNA increases the frequency of selenocysteine incorporation and MuLV UAG redefinition relative to termination. (a) Schematic representation of the reporter messages: P3 contains a single UGA codon and MuLV contains a single MuLV UAG codon and pseudoknot stimulator of read-through inserted into pDlucS1S2. (b) ^{35}S -labeled proteins resulting from RRL transcription and translation reactions supplemented with SBP2-CT and the indicated amounts ($\mu\text{g}/\mu\text{l}$ RRL reaction) of Luciferase T7 plasmid. The messages translated were as follows: lanes 1–4, P3; lanes 5–8, MuLV. The positions for protein products arising from termination at the UAG codon, the UGA codon, and the FL protein are indicated by arrows. (c) Quantitative analysis of the fold increase in selenocysteine incorporation or read-through efficiency at the UGA or UAG codons, respectively ($n=4$).

UGA codons in a single mRNA with sufficient processivity to produce FL protein?

Of central importance to this question is how the information in the 3' UTR is conveyed to the ribosome during decoding of the UGA codons. Reprogramming of the ribosome to decode UGA as selenocysteine may occur during decoding of the first UGA codon whereby the SECIS element interacts with the ribosome via L30, SBP2, or factors that are yet to be identified. The initial interaction might be facilitated by ribosome pausing or possibly by *cis*-acting elements, such as the SREs that reside near the UGA codon. Alternatively, it has been proposed that functional circularization of mRNAs through interactions between polyA binding protein and initiation factors may position the SECIS elements to interact with the ribosome early in translation, perhaps even during initiation. Intriguingly, the protein eIF4a3 shares homology to the translation initiation factor eIF4a1 and was recently shown to be a SECIS binding protein.¹⁵ Once the requisite interaction between the SECIS complex and ribosome has occurred, either the SECIS element and associated factors may remain associated with the ribosome during decoding of each UGA codon or the ribosome may be reprogrammed such that it maintains an altered state competent for redefinition of UGA codons without continued association of the SECIS. Either persistence of the SECIS complex association with the ribosome or a SECIS-independent altered conformational state of the ribosome could account for highly processive selenocysteine incorporation at downstream UGA codons by ribosomes that transit the first UGA.

To terminate or incorporate

An increase in processivity could arise due to a "loss-of-function" event in which the ribosome's ability to terminate translation is reduced, thereby favoring selenocysteine incorporation. The data presented in Fig. 1 demonstrate that after decoding of the first UGA codon in the message, a dramatic increase in selenocysteine incorporation efficiency is observed at a second downstream UGA codon. However, when the second UGA is changed to either a UAG or a UAA codon, termination of translation is highly efficient. This result indicates that the ribosome's ability to terminate translation remains intact and that, consequently, a general reduction in termination efficiency is unlikely to account for enhanced selenocysteine incorporation at downstream UGA codons.

Role of the SECIS element

Selp genes are the only known selenoprotein genes to contain two SECIS elements, suggestive of a specialized role for each SECIS element during decoding of multiple UGA codons. However, when the two SECIS elements are replaced

with SECIS1 from Selp, there was no significant change in the efficiency of selenocysteine incorporation at downstream UGA codons (Stoytcheva *et al.*²⁴ and this study). The reason that two SECIS elements are conserved in Selp genes when one will suffice is unknown and requires further study. When the Selp SECIS elements were replaced with a single SECIS element from other selenoprotein genes, varying efficiencies of selenocysteine incorporation were observed at the first UGA. A key observation in this experiment was that the increased efficiency of selenocysteine incorporation at the second UGA correlated directly with and was about 10-fold higher than the read-through efficiency at the first UGA (Fig. 8). As the second UGA codon is only decoded by ribosomes that do not terminate at the first UGA codon, this result demonstrates that the increase in processivity is observed regardless of which SECIS element is present. However, the absolute efficiency of incorporation at the downstream UGAs depends on the inherent ability of each SECIS element to deliver eEFSec and its associate tRNA to the ribosome.

The dependence of selenocysteine incorporation efficiency at *both* UGA codons on the identity of the SECIS element strongly suggests that the SECIS element interacts with the ribosome during decoding of each UGA codon in the message and does not support a model in which the SECIS element acts by a singular event that recruits or creates a population of ribosomes programmed for selenocysteine incorporation without continued association with the SECIS element. If the latter were the case, then one would expect selenocysteine incorporation at the second UGA to be equal regardless of the identity of the SECIS element; that is, all ribosomes that incorporated selenocysteine at the first UGA would have an equal probability of incorporating selenocysteine at the second and all subsequent UGA codons.

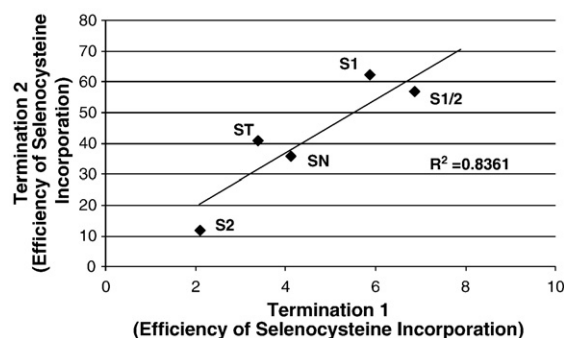


Fig. 8. Correlation of selenocysteine incorporation at the first and second UGA codons. The data from Fig. 2 are shown with the efficiency of selenocysteine incorporation at the first UGA (Termination 1) on the *x*-axis and the efficiency of selenocysteine incorporation at the second UGA on the *y*-axis (Termination 2). S1, S1/2, S2, ST, and SN represent the data from analysis of the protein products produced from P2/3S1, P2/3S1S2, P2/3S2, P2/3ST, and P2/3SN, respectively.

Sequence context effects

The sequence context of UGA codons can modulate selenocysteine incorporation rates.^{4,28–30} In this study, UGA codons were placed in a random sequence context and selenocysteine incorporation rates were shown to be as high or higher (70%–90%; Fig. 6) than those found using UGA codons in a SelP context. However, a phylogenetically conserved stem-loop structure, a potential SRE, was found downstream of the first UGA codon in the P2/3 constructs (data not shown). Deletion of sequences comprising the 3' side of the stem had a measurable effect on selenocysteine insertion at the second UGA codon (from 66% to 57%). These results collectively indicate that special sequence signals from SelP are not required for, but most likely modulate, the increase in selenocysteine incorporation efficiency following decoding of the first UGA codon.

Implications for SelP protein expression

SelP consists of two domains, an amino-terminal domain containing the first selenocysteine residue with redox function and a C-terminal domain containing multiple selenocysteine residues necessary for the delivery of selenium through the plasma to various tissues. Purification of SelP from rat plasma revealed several isoforms of the protein. These isoforms were shown by carboxypeptidase sequencing²² and mass spectrometry²³ to comprise FL and several proteins that were prematurely UGA terminated. Figures 3 and 4 demonstrate that selenocysteine incorporation efficiency at downstream UGA codons is reduced as the distance between the UGA codons is increased. This result may explain why the second and all subsequent UGA codons in SelP genes from a variety of organisms are found in close proximity near the C-terminal end of the open-reading frame. If the UGA codons were positioned far apart, selenocysteine incorporation efficiency may not be sufficient to produce FL protein.

Analysis of SelP isoforms following transfection of the zebrafish gene into cultured cells indicated that there is significant termination at the first and second UGA codons but not at the 15 subsequent UGA codons.²⁴ In the zebrafish SelP gene, the distance between the first and second UGA codons is 621 nt, which, in accord with our data, is a sufficient distance to reduce selenocysteine insertion efficiency at the second UGA codon by as much as 50%. In contrast, the second UGA and all remaining downstream UGA codons lie close together within a 291-nt region of the open-reading frame. Our data provide a viable explanation for the results observed by Stoytcheva *et al.*²⁴—that is, efficient termination at the first and second UGA codons with processive selenocysteine incorporation at the remaining UGA codons.

It is notable that zebrafish have a second SelP gene that has homology to the FL SelP gene lacking only the C-terminal domain containing the last 16 selenocysteine residues. Likewise, one isoform of SelP isolated from rats corresponded to termination at the second

UGA codon. Based on these observations, it is possible that termination at the second UGA codon could produce a functional isoform of SelP containing a single selenocysteine residue with redox activity. Further studies are needed to address the potential redox function and biological role of this isoform.

Processive selenocysteine incorporation

Based on the results presented here, we propose a simplified model for highly processive selenocysteine incorporation that invokes properties intrinsic to the mechanism of selenocysteine incorporation. In this model, the SECIS complex is loaded onto the ribosome prior to or during decoding of the first UGA codon. Once assembled, the ribosome and associated SECIS complex proceed to the next UGA codon primed for highly efficient selenocysteine incorporation. As discussed above, the dependence of selenocysteine incorporation efficiency at the first and downstream UGA codons on the identity of the SECIS element (Figs. 2 and 8) argues that the SECIS is interacting with the ribosome at each UGA in the message. Although it cannot be strictly ruled out that the SECIS complex dissociates and then reassociates with the ribosome at each UGA codon, the simplest model is one in which the SECIS element and associated factors track with the ribosome following selenocysteine insertion at the first UGA and remain associated during decoding of all subsequent UGAs. In this scenario, only recycling of eEFSec with an amino-acylated tRNA would be required for efficient selenocysteine incorporation.

One important implication of this model is that only one ribosome at a time can successfully translate through multiple UGA codons to produce FL protein. Meanwhile, any ribosomes translating to the first UGA codon will terminate. Only when the SECIS-associated ribosome has completed translation of the message can the SECIS element be recycled to a new ribosome. This aspect of the model explains why the first UGA and messages with only one UGA have low selenocysteine incorporation efficiency relative to termination. This model is also consistent with the observation that increasing the distance between UGAs diminishes selenocysteine incorporation efficiency at the second UGA codon (Fig. 3). With increased codons to decode between the UGAs, the SECIS complex would have more time to inadvertently disassociate from the ribosome.

Reducing the number of ribosomes translating to the first UGA codon either by inclusion of an upstream MuLV read-through cassette or by addition of a competing mRNA (Fig. 7) results in a higher percentage of ribosomes incorporating selenocysteine. One explanation for this result is that fewer ribosomes encounter the first UGA during the time it takes the SECIS-associated ribosome to complete translation of the message and for the SECIS complex to reassociate with an upstream ribosome. This observation suggests that selenocysteine incorporation efficiency *in vivo* may be

controlled by altering the number of ribosomes that initiate translation on selenoprotein messages.

Finally, the finding that the ability to decode multiple UGAs as selenocysteine is not unique to SelP sequences demonstrates that heterologous non-selenoprotein genes can be engineered to express proteins containing multiple selenocysteine residues.

Materials and Methods

Construction of pDluc reporter plasmid

The reporter plasmid pDluc was constructed by inserting the firefly luciferase coding sequence from pGL4.10 (Promega) into the XbaI restriction site of pGL4.73 (Promega) downstream of the Renilla luciferase gene. A T7 promoter was inserted into the HindIII restriction site upstream of the Renilla gene. The region between the Renilla and firefly luciferase genes was altered to include XhoI, HindIII, and BglII cloning sites. In addition, the native ATG start codon of firefly was changed to ATT. Further details are available upon request.

Insertion of SECIS elements into pDluc

The following SECIS sequences were inserted into the XbaI site located downstream of the firefly luciferase gene in pDluc:

SECIS1 (S1): GGTTAATATGCTTTTTTTTCTTTTC-CAGTGTCTATTTGCTTTAATGAGAATAGAAACG-TAAACTATGACCTAGGGGTTTCTGTTGGATAAT-TAGCAGTTTAGAATGGAGGAAGAACAACAAA-GAC

SECIS2 (S2): TTGTCTGTCTCTATATTGCTTAG-TAAGTATTTCCATAGTCAATGATGGTTTAA-TAGGTAACCAAAACCTATAAACCT-GACCTCCTTTATGGTTAATACTATTAAGCAA-GAATG

SECIS1 and SECIS2 (S1/S2): GGTTAATATGCTTTTT-TTTTCTTTTCCAGTGTCTATTTGCTTTAATGAG-AATAGAAACGTAAACTATGACCTAGGGGTTTCTGTTGGATAATTAGCAGTTTAGAATGGAGGAAG-AACAACAAGACATGCTTTCCATTTTTTCTTTA-CTTATCTCTCAAAACAATACTTTGTC-TTTTCAATCTTCTACTTTTAACTAATAAAA-TAAGTGGATTTGTATTTAAGATCCAGAAA-TACTTAACACGTGAATATTTTGCTAAAAAAGCA-TATATAACTATTTTAAATATCCATTTATCTTTTG-TATATCTAAGACTCATCTGATTTTACTATCA-CACATGAATAAAGCCTTTGTATCTTTCTTCTC-TAATGTTGTATCATACTCTTCTAAAACCTT-GAGTGGCTGTCTTAAAAGATATAAGGGGAAA-GATAATATGTCTGTCTCTATATTGCTTAGTAAG-TATTTCCATAGTCAATGATGGTTTAAATAGG-TAAACCAACCTATAAACGACCTCCTT-TATGGTTAATACTATTAAGCAAGAATG

SelN SECIS (SN): AGTGGCTTCCCCGGCAG-CAGCCCCATGATGGCTGAATCCGAAATCCTC-GATGGGTCCAGCTTGATGTCTTTCAGCTG-CACCTATGGG

SelT SECIS (ST): GATCATTGCAAGAGCAGCGT-GACTGACATTATGAAGGCTGTACTGAAGACAG-CAAGCTGTAGTACAGACCAGATGCTTTCTTGG-CAGGCTCGTTGTACCTCT

Sequences containing one or more UGA codons were inserted into the respective pDluc vectors described in the text between the XhoI and BglII sites. Sequences are shown below. In-frame TGA, TAG, and TAA codons and their respective changes are underlined.

Reporter constructs

SelP-derived sequence inserts:

P1: CTCCAATGGTTCAGTGACTGTGGTGTCTTCTT-CAAGCCAGCTGATACCTGTGCATACTGCAGGCATC-TAAATTAGAAGACCTGCGAGTAAACTGAAGAAA-GAAGGATATCTAATATTCTTATATT

P2: ACTCTGTAAATTGCCCCACAGATTCA-GAGTTGGCTCCTAGGAGCTGATGCTGCCATTGTC-GACATCTGATATTTGAAAAAACAGGGTCTG-CAATCACCTGGCAGTGTAAGAAAAACCTCC-CATCTTTATGT

P3: ACTCTGTAAATTGCCCCACAGATTCA-GAGTTGGCTCCTAGGAGCTGATGCTGCCATTGTC-CATTGTCGACATCTGATATTTGAAAAAACAGGGTCTGCAATCACCTGACAGTGTAAGAAAAACCTCCCATCTTTATGT

P2/3: ACTCTGTAAATTGCCCCACAGATTCA-GAGTTGGCTCCTAGGAGCTGATGCTGCCATTGTC-GACATCTGATATTTGAAAAAACAGGGTCTG-CAATCACCTGACAGTGTAAGAAAAACCTCC-CATCTTTATGT

The constructs P3, P2, P2/3UAG and P2/3UAA all contained the same sequence insert as for SelP2/3 with the exceptions that in P3, the first UGA in the SelP2/3 insert was changed to TGG, and in SelP2, SelP2/3UAG, and SelP2/3UAA, the second UGA in the SelP2/3 insert was changed to a CGA, TAG, or TAA, respectively.

P2/10: ACTCTGTAAATTGCCCCACAGATTCA-GAGTTGGCTCCTAGGAGCTGATGCTGCCATTGTC-GACATCTGATATTTGAAAAAACAGGGTCTG-CAATCACCGACAGTGTAAGAAAAACCTCC-CATCTTTATGTAGCCGACAGGGACTTCGGGCA-GAGGAGAACATAACTGAATCTTGTTCAGC-GACGTTTGCTCCAGCTGCCCGACAAATAAGT-CAGCAGCTTATACCCACAGAAGCCAGTGC-CAGTCGACGCCGAAAGATCAGGCAAAAAAGC-GAGAATGACCTTCAAAC

In order to examine the effect of varying the distance between the first and second UGA codons in the SelP message, we cloned sequences from the SelP gene starting just after the initiation ATG codon and including the first TAG into the XhoI site of P2/10 described above. Sequential deletions were made in the region between the first and second TGAs. The sequences of the longest insert is as follows:

P726: TGGAGAAGCCTGGGGCTTGCCCTGGC-TCTCTGTCTCCTCCCATCGGGAGGAACAGA-GAGCCAGGACCAAAGCTCCTTATGTAAG-CAACCCCCAGCCTGGAGCATAAGAGATCAA-GATCCAACGCTAAACTCCAATGGTTCAGT-GACTGTGGTTGCTCTTCTTCAAGCCAGCTGA-TACCTGTGCATACTGCAGGCATCTAAATTAGAA-GACCTGCGAGTAAAACTGAAGAAAGAAAGGA-TATTCTAATATTTCTTATATTGTTGTTAATCAT-CAAGGAATCTCTTCTCGATTAAATAACACA-CATCTTAAGAATAAGGTTTCAGAGCATATTCTT-

GTTTATCAACAAGAAGAAAACCAAACAGATGT-
CTGGACTCTTTTAAATGGAAGCAAAGAT-
GACTTCTCATATATGATAGATGTGGCGTCTT-
GTATATCATCTTGGTTTGCCTTTTTCCTTCC-
TAACCTTCCCATATGTAGAAGAAGCCATTAA-
GATTGCTTACTGTGAAAAGAAATGTG-
GAAACTGCTCTCACGACTCTCAAAGATGAA-
GACTTTTGTAACGTGTATCTTTGGCTACTGTG-
GATAAAACAGTTGAAACTCCATCGCCTCATTAC-
CATCATGAGCATCATCACAATCATGGACATCAG-
CACCTTGGCAGCAGTGAGCTTTCAGAGAAT-
CAGCAACCAGGAGCACCAAAATGCTCCTACT-
CATCTGCTCCTCCAGGCCTTCATCACACCA-
TAAGCACAAGGGTCAGCATAGGCAGGGTCACC-
CAGAGAACCGAGATATGCCAGCAAGTGAA-
GATTTACAAGATTTACAAAAGAAGCTCTGTC-
GAAAGAGATGTATAAATCAATT

The first 647 nt, 443 nt, or 227 nt of this sequence were cloned into the Xho-1 site of P2/10 to create P516, P312, and P96, respectively.

The effect of distance between UGA codons was also investigated by inserting random sequence between TGA2 and TGA3 in our SelP2/3 constructs. Those constructs are as follows:

P2/3_78: ACTCTGTAAATTGCCCACAGATTCA-
GAGTTGGCTCCTAGGAGCTGATGCTGCCATTGTC-
GACATCTGATATTTGAAAAACAGGGTCTGCAA-
TATCGGATAGGAAATCGTTACAATCGATCACCT-
GACAGTGTAAGAAAAACCTCCCATCTTTATGT
P2/3_108: ACTCTGTAAATTGCCCACAGATTCA-
AGTTGGCTCCTAGGAGCTGATGCTGCCATTGTC-
GACATCTGATATTTGAAAAACAGGGTCTGCA-
ATATCGGATAGGCAAAATCGTTACAATCGATATC-
GATATCGATATCGATCAAGCTTACCACACCTGA-
CAGTGTAAGAAAAACCTCCCATCTTTATGT

Non-SelP-based sequence inserts

4UGA: GTCGACGCAGAGACAATGACCGAATCA-
GGACTTACGGATCCATGGGCTTCATAGACCACT-
CAAGTACGGCAGGCCATGAGAGCTAAGC-
GACTCCAAGCGAAATTTCCAGGGCATATTTT-
TAGGCCAATACCGGACGCCATACTTGTGGCATT-
TCCCAATTGGCATATGAGGGCCCCAATACAGAC-
AAAAGCTTGACGGACTAGGAATCTTCGC-
CCAGGATCCCCGAAAAGCGGCCAATTATAGC-
GCCGAACCTAGGGCACGGCACAGTCGCGATCTT-
ACCAGGCTGATCGCTACGTTGGGACGGATCC

The 2UGA and 1UGA/UAA constructs contain the same sequence as 4UGA with the second and third TGAs changed into ATT and TAC respectively. In addition, in the 1UGA/UAA construct, the last TGA was changed to a TAA.

MuLV-containing sequence inserts

MuLV/P3: ACTGACCCTAGATGACTAGGGAGGTC-
AGGGTCAGGAGCCCCCCCCCTGAACCCAGGATAA-
CCCTCAAAGTCGGGGGGCAACCCGGTCGACATC-
TGATATTTGAAAAACAGGGTCTGCAATCACCT-
GACAGTGTAAGAAAAACCTCCCATCTTTATGT
MuLV/CAG/P3: ACTGACCCTAGATGACCAGGG-
AGGTCAGGGTCAGGAGCCCCCCCCCTGAACC-
CAGGATAACCCCTCAAAGTCGGGGGGG-
CAACCCGGTCGACATCTGATATTTGAAAAAA-

CAGGGTCTGCAATCACCTGACAGTGTAAG-
GAAAACCTCCCATCTTTATGT

MuLV/MuLV: ACTGACCCTAGATGACTAGG-
GAGGTCAGGGTCAGGAGCCCCCCCCCTGAACC-
CAGGATAACCCCTCAAAGTCGGGGGGG-
CAACCCGGTCGACATCTGATATTTACACGTG-
GACTGACCCTAGATGACTAGGGAGGTCAGGGT-
CAGGAGCCCCCCCCCTGAACCCAGGATAACCCCT-
CAAAGTCGGGGGGCAACCCGGA

MuLV: GACCCTAGATGACTAGGGAGGTCAGGGT-
CAGGAGCCCCCCCCCTGAACCCAGGATAACCCCT-
CAAAGTCGGGGGGCAACCCGTCACC

RRL transcription and translation

Rabbit reticulocyte translation reactions using Promega's TNT Quick Coupled Transcription/Translation System were carried out according to the manufacturer's instructions in the presence of L-[³⁵S]methionine, in the presence or absence of saturating levels of purified SBP2-CT as described by Howard *et al.*⁵ In competition reactions, the plasmid Luciferase T7 (Promega) was included at the concentrations indicated and reporter DNAs were reduced by 4-fold to 0.005 µg/µl. ³⁵S-labeled protein products were separated by electrophoresis on 10% Bis-Tris NuPAGE denaturing gels in Mops buffer according to the manufacturer's specifications (Invitrogen). Gels were fixed in 50% methanol:7.5% acetic acid and dried. Radiolabeled ³⁵S-labeled protein bands were visualized using the Typhoon PhosphorImager (GE Healthcare), and band intensities were quantified using ImageQuant software.

Calculating the efficiency of selenocysteine insertion

The intensity of all protein bands resulting from termination and the FL protein were determined using ImageQuant software, and then each value was corrected for the number of methionines. To calculate the percentage of termination, we divided the intensity of the band corresponding to termination at that position by the sum of that band and all bands arising from downstream termination at UGA (or UAG and UAA) codons. This value was then subtracted from 100 to determine the efficiency of selenocysteine incorporation (or UAG redefinition) as a percentage of ribosomes which encountered that UGA (UAG) codon. Each number represents the average value from at least two independent experiments ($n=6$, except where indicated), error bars represent the standard deviation from the mean, and p values were calculated by Student's t test.

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