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Functional Analysis of the Interplay between Translation Termination, Selenocysteine Codon Context, and Selenocysteine Insertion Sequence-binding Protein 2*

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Malavika Gupta and Paul R. Copeland[1]

From the Department of Molecular Genetics, Microbiology and Immunology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

A selenocysteine insertion sequence (SECIS) element in the 3′-untranslated region and an in-frame UGA codon are the requisite *cis*-acting elements for the incorporation of selenocysteine into selenoproteins. Equally important are the *trans*-acting factors SBP2, Sec-tRNA$^{[Ser]Sec}$, and eEFSec. Multiple in-frame UGAs and two SECIS elements make the mRNA encoding selenoprotein P (Sel P) unique. To study the role of codon context in determining the efficiency of UGA readthrough at each of the 10 rat Sel P Sec codons, we individually cloned 27-nucleotide-long fragments representing each UGA codon context into a luciferase reporter construct harboring both Sel P SECIS elements. Significant differences, spanning an 8-fold range of UGA readthrough efficiency, were observed, but these differences were dramatically reduced in the presence of excess SBP2. Mutational analysis of the "fourth base" of contexts 1 and 5 revealed that only the latter followed the established rules for hierarchy of translation termination. In addition, mutations in either or both of the Sel P SECIS elements resulted in differential effects on UGA readthrough. Interestingly, even when both SECIS elements harbored a mutation of the core region required for Sec incorporation, context 5 retained a significantly higher level of readthrough than context 1. We also show that SBP2-dependent Sec incorporation is able to repress G418-induced UGA readthrough as well as eRF1-induced stimulation of termination. We conclude that a large codon context forms a *cis*-element that works together with Sec incorporation factors to determine readthrough efficiency.

Selenocysteine (Sec),[2] the 21st amino acid, is incorporated into proteins via a recoding of the UGA stop codon (1). Selenoproteins are primarily involved in protecting the cell from oxidative stress, and the concerted effort of several protein factors and RNA elements is required for the production of these proteins (2). Two *cis*-elements in the mRNA are required for the incorporation of selenocysteine into a nascent polypeptide. These are an in-frame UGA codon and a structure called the SECIS (Sec insertion sequence) element (3). The SECIS element is a stem loop structure consisting of a core stem and a terminal bulge or loop. The SECIS core is comprised of an AUGA motif positioned opposite a GA dinucleotide forming a non-Watson-Crick base-paired quartet, thus making this RNA a member of the kink-turn family of RNA motifs (3). The terminus of the SECIS stem consists of either a 9–11-nucleotide loop (designated Form 1) or a 5′ bulge followed by a smaller 6-nucleotide loop (designated Form 2). Both SECIS forms contain a conserved AAR motif within the loop or bulge, respectively (4). SECIS binding protein 2 (SBP2) (5), a Sec-specific translation elongation factor eEFSec (6), and a Sec-tRNA$^{[Ser]Sec}$ (7) are the three *trans*-acting factors that have been identified to be essential for Sec incorporation. Recently ribosomal protein L30 has been found to interact with the SECIS element but whether it is required for Sec incorporation remains to be determined (8).

Most selenoprotein mRNAs contain only a single Sec codon and a single SECIS element. The selenoprotein P (Sel P) mRNA is unique because it contains multiple Sec codons ranging from 10 in mammals to a maximum of 18 in some amphibians (9, 10), and it has two SECIS elements (11). In addition to termination at its naturally occurring stop codon, rat Sel P has protein isoforms resulting from termination at the second, third, and seventh UGAs (12). The *in vivo* frequency of translation termination at these UGAs is not known, but these isoforms have been observed in preparations of Sel P purified from rat serum (12). The role of the dual SECIS elements has only recently been studied in detail. In 2006 Stoytcheva *et al.* (13) determined that the second SECIS element of zebrafish Sel P was required for readthrough of the first UGA and the first SECIS element was necessary for readthrough of subsequent UGAs and consequently, production of full-length protein (13). However, the mechanistic basis for this distinction of function remains elusive.

Studies in bacteria and eukaryotes have shown that translation termination is influenced by the bases near the stop codon, with a strong bias toward nucleotides 3′ of the stop codon (14–16). These findings support the model that translation termination is dictated by more than three residues with the identity of the base at the fourth position (+4 position; hereafter referred to as the "fourth base") contributing to the efficiency of translation termination. In several organisms including *Escherichia coli* and mammals, the strength of a translation termina-

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[1] To whom correspondence should be addressed: 675 Hoes Lane, Piscataway, NJ 08854. Fax: 732-235-5223; E-mail: paul.copeland@umdnj.edu.

[2] The abbreviations used are: Sec, selenocysteine; SBP2, Sec insertion sequence binding protein 2; UTR, untranslated region; RRL, rabbit reticulocyte lysate.

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tion context follows the order of A > G > C > U, where an mRNA with a stop codon followed by an A is terminated more efficiently than one with a U at the same position (16–18). More recent studies have shown that translation termination is determined by a combination of the identity of the stop codon and the nucleotide at the fourth base (16, 17, 19). Studies have shown the ratio of Sec incorporation to termination also to be affected by the base immediately 3′ of the Sec codon. The rules for termination at a Sec codon differ from the A > G > C > U rule observed in non-Sec codons in mammals and *E. coli*. A study examining the effect of the fourth base on the porcine phospholipid hydroperoxide glutathione peroxidase UGA readthrough found it to be highest with the native fourth base, a G (20). A gradual decrease in readthrough was observed when the fourth base was mutated to a C or a U. An A at the fourth base of the same construct resulted in the most drastic reduction in readthrough (20). A separate study of Sec incorporation in a Type 1 deiodinase reporter showed that when the Sec codon was followed by a strong termination context, an A as the fourth base, readthrough was much greater than when the codon was followed by any of the remaining three bases (21). These previous studies have contributed to the model that the efficiency of Sec incorporation is not simply inversely proportional to the efficiency of translation termination, suggesting that other determinants are required for processive and efficient Sec incorporation.

Previously we showed that *in vitro*, Sec incorporation in a monocistronic luciferase reporter bearing the rat phospholipid hydroperoxide glutathione peroxidase SECIS element occurs with a maximum of ~8% efficiency. This contrasted with an ~40% efficiency when full-length Sel P was translated in the same system, suggesting that one or more elements within the Sel P mRNA sequence were providing increased efficiency (22). This study determines how Sel P sequences contribute to the efficiency of Sec incorporation and its interplay with translation termination.

EXPERIMENTAL PROCEDURES

Codon Context Plasmid Construction—A firefly luciferase construct containing an in-frame UGA at position 258 was created as previously described (22). The coding region of this construct was further mutated, 12 nucleotides upstream and downstream of UGA 258, to create AflIII and SgfI restriction sites. PacI and NotI linkers were used to clone the first 770 nucleotides of the Sel P 3′-UTR downstream of the Luc UGA open reading frame. This stretch of 3′-UTR contained both SECIS elements. Oligos, 27 nucleotides long and containing each Sel P UGA codon and its context (12 nucleotides flanking on either side), were synthesized with AflIII and SgfI linkers. These oligos were then phosphorylated and ligated into the firefly luciferase vector pre-digested with AflIII and SgfI. The reading frame of the original firefly luciferase construct was not shifted by cloning the 27-nucleotide Sel P oligos. All constructs were in pcDNA3.1 (Invitrogen) and regulated by the bacteriophage T7 promoter.

Generation of Point Mutants—Point mutants were created using site-directed mutagenesis (QuikChange, Stratagene) as per the manufacturer's protocol. Mutants were verified by automated DNA sequencing.

In Vitro Transcription and Translation—Constructs were linearized with either XhoI or PacI. XhoI digestion generated a template with a 3′-UTR downstream of the T7 promoter, allowing transcription of the coding region and the 770 nucleotides of the Sel P 3′-UTR. PacI digestion created a template that had the 3′-UTR, including the SECIS elements, upstream of the T7 promoter, thereby allowing transcription of only the coding region and not the 3′-UTR. The eRF1 and eEFSec constructs were digested with XbaI and HindIII, respectively. Linearized DNA was then transcribed with the T7 mMessage kit (Ambion). Rabbit reticulocyte lysate (Promega) was used for *in vitro* translations. *In vitro* translations were performed with [^{35}S]Met according to the manufacturer's protocol and three separate translations were done for each construct. Briefly, 12.5-μl reactions contained 8 μl of rabbit reticulocyte lysate (RRL), 0.25 μl of 40 units/μl of RNasin (Promega), 0.25 μl of 1 mM amino acid mixture minus methionine, 0.5 μl of [^{35}S]Met at 10 mCi/ml, 100 ng of reporter mRNA and varying amounts of CTSBP2 or 1× phosphate-buffered saline and 2 mM dithiothreitol. The amount of CTSBP2 added to the reactions was either 0.2 or 2 pmol, but the total volume of CTSBP2 was 3 μl. CTSBP2 was diluted in 1× phosphate-buffered saline and 2 mM dithiothreitol. Two microliters of the translation reactions were resolved by 12% SDS-PAGE and quantitated by PhosphorImager analysis. As translations carried out in the absence of added SBP2 yielded a full-length product of low intensity, these reactions were exposed for up to 8 days. An overnight exposure was sufficient for reactions carried out with CTSBP2. To quantitate the bands, a rectangle or a polygon was drawn around each full-length and UGA-termination product. The background was subtracted by copying and pasting the rectangles or polygons directly above our product of interest. For co-translation experiments, 25 ng of human eRF1 and 100 ng of mouse eEFSec mRNAs were translated with the specified Sel P contexts.

^{75}Se Labeling—Selenium 75 (400 μM at 380 mCi/mg), in the form of selenous acid (obtained from the University of Missouri Research Reactor) was first neutralized with NaOH and diluted to a working concentration of 0.8 μM. The final concentration of ^{75}Se in our reactions was 0.064 μM. Except for the addition of ^{75}Se, *in vitro* translations were carried out exactly as explained above. After the 1-h incubation, 16% of the translation reactions were resolved by 12% SDS-PAGE and quantitated by PhosphorImager analysis. To account for potential differences in sample loading, the SDS gel was stained with Gel Code Blue (Pierce) and an unknown protein consistently present in each sample was used to normalize the ^{75}Se-labeled product.

Preparation of CTSBP2—Recombinant Xpress/His-tagged CTSBP2 was made as previously described (23).

Bioinformatic Analysis—Mouse, human, bovine, and orangutan Sel P Sec codons and their 12 flanking nucleotides on either side were aligned to the corresponding conserved Sec codons and their contexts in rat Sel P. WebLogo was used to graph homology (29).

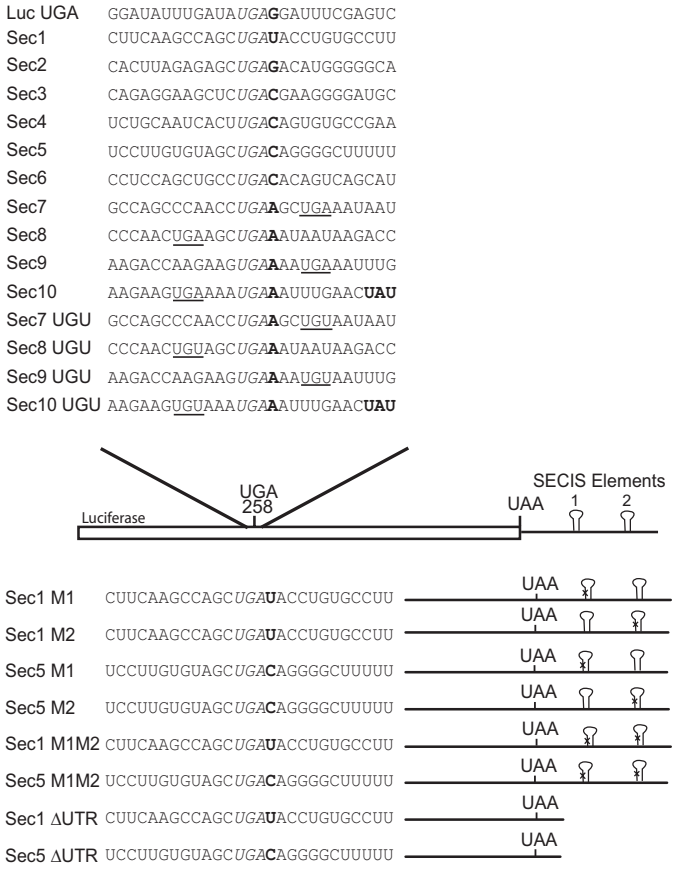

FIGURE 1. **Schematic representation of constructs containing rat Sel P Sec codon contexts.** Each rat Sel P codon context replaced the 24 nucleotides surrounding codon 258 of Luc UGA. With the exception of ΔUTR constructs, all had 770 nucleotides of the Sel P 3′-UTR and both of its SECIS elements. *M1*, *M2*, and *M1M2* indicate mutations in the first, second, or both SECIS elements, respectively. Fourth base of all constructs is shown in *bold*, the Sec codon is *italicized*, and overlapping Sec codons (in Sec7 UGU-Sec10 UGU) are *underlined*. All constructs had the Luc UGA UAA stop codon. The natural Sel P UAA stop codon is mutated to the UAU tyrosine codon, also shown in *bold*.

RESULTS

Sel P Codon Contexts—To understand the impact of codon context on Sec incorporation, we used each of the 10 rat Sel P UGAs, along with their four flanking codons on both sides, replacing the corresponding sequence in the open reading frame of a firefly luciferase construct (Fig. 1, *Sec1–Sec10*). This monocistronic luciferase construct contained an in-frame UGA at codon 258 (Fig. 1, *Luc UGA*). A Sec codon at this position had been previously shown to permit Sec incorporation at an efficiency of ~5–8% (22). In addition to the Sel P UGA codons and their native codon contexts, our constructs also contained the first 770 nucleotides of the rat Sel P 3′-UTR including both of its SECIS elements (Fig. 1). In rat Sel P, the seventh to the 10th Sec codons are closely clustered. Consequently, Sec constructs 7–10 contained more than one in-frame UGA and common or overlapping nucleotides. The overlapping UGAs, which are *underlined* in Fig. 1, were mutated to the UGU cysteine codon, thus generating constructs designated Sec UGU. Sec10 was further mutated to change the natural rat Sel P stop codon, UAA, to the tyrosine UAU codon, enabling us to assess its readthrough ability. As a control, we used the parent firefly luciferase construct with the in-frame UGA at amino acid 258 (Fig. 1, *Luc UGA*). Like the Sec constructs, the Luc UGA construct also contained the first 770 nucleotides of the rat Sel P 3′-UTR including both of the rat Sel P SECIS elements.

Because RRL contains all of the factors so far identified to be required for Sec incorporation (5, 22), we used this system to assess any differences in readthrough of the Sel P codon contexts. As expected, all of our *in vitro* translation reactions produced two predominant products. The larger product corresponded to the full-length polypeptide resulting from UGA readthrough, whereas the smaller product was the pre-Sec peptide resulting from termination at the UGA codon. The efficiency of translation for each construct was reported as the percent readthrough calculated by dividing the amount of full-length polypeptide produced by the sum of the full-length and pre-Sec peptides. Most of the experiments make use of [^{35}S]Met to label both termination and readthrough products so that we can evaluate both nonspecific readthrough as well as that resulting from Sec incorporation. Because Sec incorporation in RRL is limiting for SBP2 (22), all of our *in vitro* translations were carried out either in the absence of added SBP2 or with recombinant rat SBP2 corresponding to the fully functional C-terminal 445 amino acids and bearing an N-terminal Xpress/His tag (CTSBP2) (22). The amount of CTSBP2 needed to reach intermediate (0.2 pmol) and saturating (2 pmol) levels of Sec incorporation was experimentally determined by using varying amounts of SBP2 in the luciferase-based Sec incorporation assay (data not shown). Assessing the efficiency of Sec incorporation in the absence of added CTSBP2 presented a challenge in that long exposures were required to acquire accurate quantitation of the readthrough product. These exposures resulted in a saturation of signal for the termination product and thus in these cases the value for efficiency was slightly overestimated. When the full-length products were normalized by comparing them to that obtained for the Luc UGA control, we calculated the level of overestimation to be a factor of 1.4 (data not shown).

Sec Incorporation in Sel P Is Regulated by Codon Context—Each Sel P codon context was first *in vitro* translated in the absence of added SBP2, thus making use of the limiting amount of endogenous SBP2 in RRL (5). Of all the Sel P contexts with a single Sec codon, Sec1 might be expected to have the greatest readthrough because it has what is classically described as the weakest context for translation termination, *i.e.* a U as the fourth base (Fig. 1). Yet, based on our *in vitro* translation data, Sec1 provided lower readthrough than Sec4–Sec6, each of which has a stronger predicted context for termination with a C at the fourth base (Fig. 2*A*, compare *lane 2* with *lanes 5–7*). In the absence of CTSBP2, several contexts provided virtually no readthrough including the Luc UGA control, Sec3, and Sec7–Sec10. Interestingly, significant differences in readthrough efficiency were observed among Sec3–Sec6, all of which share a C residue at the fourth base. Sec3 was ~5-fold less efficient at readthrough than Sec6 and 8-fold less efficient than Sec4 and Sec5 (Fig. 2*A* compare *lanes 4–7*). The striking lack of readthrough for Sec7–Sec10 with endogenous SBP2 is likely due to the presence of dual UGA codons in each of these contexts. A graphical representation of the amount of readthrough

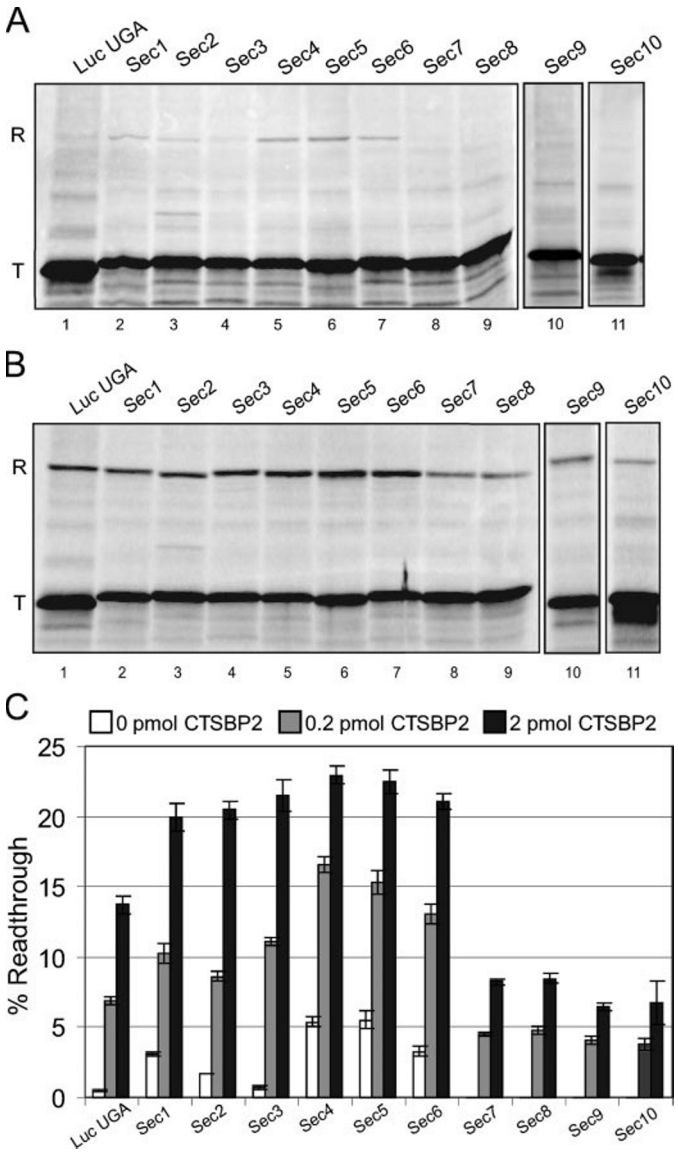

FIGURE 2. ***In vitro* translation of Sec1–Sec10.** *A*, Sel P codon context constructs were translated in the presence of endogenous SBP2. The full-length peptide (*R*) that was the product of UGA readthrough, and the pre-Sec peptide (*T*) resulting from termination at the UGA, are labeled. *B*, translation reactions of Sec1–Sec10 were supplemented with 0.2 pmol of CTSBP2. *C*, graphical representation of data in *A* and *B*, indicated by the *white* and *gray bars*, respectively, as well as readthrough in the presence of 2 pmol of CTSBP2 (*black bars*). All data represent the average ± S.E. from at least three experiments.

supported by the Sel P UGA codon contexts is shown in Fig. 2*C* (*white bars*). Fig. 2, *A* and *C*, clearly show that the Sel P Sec codon contexts had readthrough efficiencies that did not correlate with the fourth base hierarchy for translation termination. These results, together with previous studies of the role of the fourth base in regulating Sec incorporation, confirm that the fourth base of a Sec codon context is not a good predictor of readthrough efficiency thus alluding to the involvement of either a larger codon context determinant, a role for the SECIS elements, or both.

To analyze the differences in readthrough efficiency under conditions where Sec incorporation levels were increased, we supplemented the translation reactions with 0.2 pmol of CTSBP2, which is in the middle of the linear range of response for the *in vitro* Sec incorporation assay (data not shown). Under these conditions, there was a 3–16-fold increase in readthrough for most constructs (Fig. 2*B*) and a general reduction in the differences in readthrough efficiency between constructs (Fig. 2*C*, *gray bars*). The Luc UGA control appears to generate a higher level of full-length product, but the amount of termination product is consistently higher. The basis for this, as well as the fact that the pre-Sec peptide runs at a slightly lower apparent molecular weight, is unknown. With 0.2 pmol of CTSBP2, average readthrough of Sec1 rose from 3.1 to 10% (compare *lane 2* in Fig. 2, *A* and *B*), whereas average readthrough for Sec3 rose almost 16-fold from 0.7 to 11% (compare *lane 4* in Fig. 2, *A* and *B*). Sec4–Sec6, all of which had the highest average readthrough with endogenous SBP2, maintained their high readthrough profile in the presence of 0.2 pmol of CTSBP2. Average readthrough of Sec4 and Sec5 rose 3-fold from ~5 to ~15%, respectively, whereas average readthrough of Sec6 rose 4-fold from 3 to 13% (compare Fig. 2, *A*, *lanes 5–7* to *B*, *lanes 5–7*). These data are shown graphically in Fig. 2*C* (*gray bars*).

Interestingly, in the contexts that contained a single Sec codon, the differences in readthrough that existed in the presence of endogenous SBP2 or 0.2 pmol of CTSBP2 were eliminated with saturating levels of SBP2. When saturating levels of SBP2 (2 pmol of CTSBP2) were added to the reactions, readthrough was again substantially increased in the constructs containing a single in-frame Sec codon (Sec1–Sec6) but to a lesser extent in the dual UGA contexts (Sec7–Sec10). Fig. 2*C* (*black bars*) shows that with saturating amounts of SBP2, average readthrough reaches its peak with an efficiency of ~20%, still below the maximum of 40% achieved with full-length Sel P *in vitro* (22). The contexts containing two Sec codons (Sec7–Sec10) yielded uniformly lower readthrough both in the presence and absence of CTSBP2 when compared with all other contexts (Fig. 2, *A* and *B*, *lanes 8–11*). With saturating amounts of SBP2, readthrough of Sec7–Sec10 was still restricted to ~7% (Fig. 2*C*, *black bars*). That excess SBP2 could not promote maximum Sec incorporation efficiency (~20%; Fig. 2*C*, *black bars*) suggested that the decoding of dual UGA codons as Sec may require other *cis*-elements present in full-length Sel P.

Sel P SECIS Elements Differentially Regulate Readthrough and Sec Incorporation—Because the addition of SBP2 to RRL reduced the differences in readthrough dictated by codon context, we set out to determine the specific contributions of readthrough resulting from Sec incorporation *versus* nonspecific readthrough (translational infidelity) using Sec1 and Sec5 as examples of low and high efficiency, respectively. As a control to test for UGA specificity, we mutated the Sec codon of Sec1 and Sec5 to the UAA stop codon. This nonsense mutation yielded no readthrough even in the presence of added SBP2 showing our readthrough was UGA-specific (data not shown). In addition, to assess the potential role of eEFsec in regulating Sec incorporation efficiency, Sec1 and Sec5 mRNAs were co-translated with 100 ng of eEFSec mRNA, which had no effect on readthrough (data not shown). This result was not unexpected because we have previously established that RRL is limiting only for SBP2 (22). To create constructs that were unable to support Sec incorporation, both SECIS elements were mutated

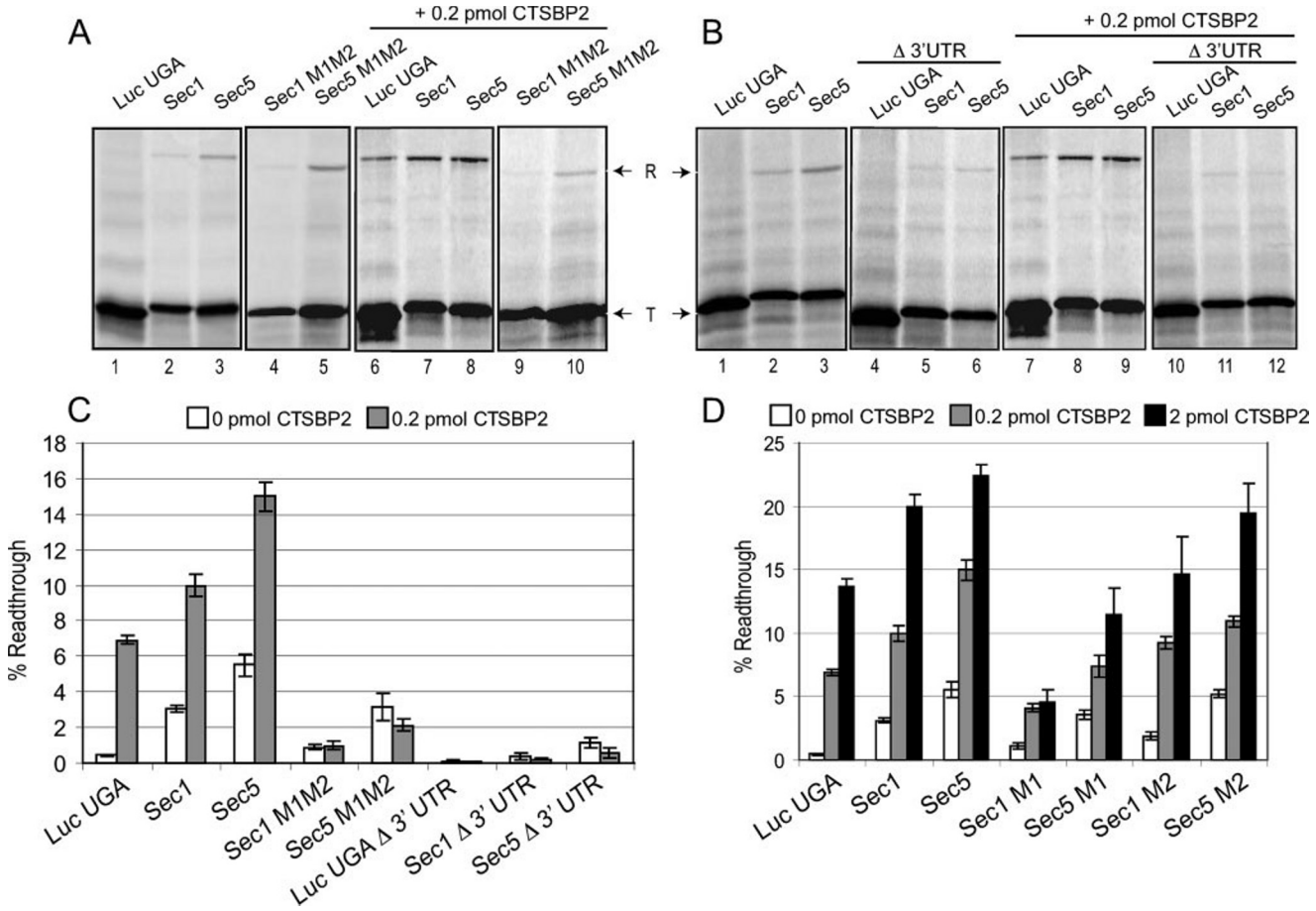

FIGURE 3. ***In vitro* translation of SECIS mutants and ΔUTR constructs.** *A*, Luc UGA, Sec1, Sec5, and Sec1 and Sec5 with double mutant SECIS elements (M1M2) were translated with endogenous SBP2 (*left panel*) or with 0.2 pmol of CTSBP2 (*right panel*). The full-length (*R*) and pre-Sec (*T*) peptides are labeled. *B*, Luc UGA, Sec1, and Sec5 possessing or lacking a 3′-UTR were translated with endogenous SBP2 (*left panel*) or with 0.2 pmol of CTSBP2 (*right panel*). *C*, graphical representation of data in *A* and *B*. *D*, graphical representation of Sec1 and Sec5 with either wild-type SECIS elements or single SECIS mutants containing point mutations (AUGA to AUCC) in the core. The *white*, *gray*, and *black bars* represent enodogenous SBP2, 0.2 pmol, and 2 pmol of CTSBP2, respectively. All experiments are shown as the mean ± S.E. of at least three experiments.

at the core region (AUGA to AUCC), a mutation that eliminates the 5′ side of the essential GA quartet and that prevents SBP2 binding and Sec incorporation for other SECIS elements (24).[3] Fig. 3*A* (*lanes 4, 5, 9*, and *10*) shows the results of translating Sec1 and Sec5 mRNAs harboring double mutant SECIS elements (M1M2) with endogenous SBP2 or 0.2 pmol of CTSBP2. With endogenous SBP2, the double mutation in Sec5 decreased readthrough from ~5 to ~3% (Fig. 3*A*, compare *lanes 3* and *5*), indicating that the efficiency of Sec incorporation is only about 2% (Fig. 3*C*, *white bars*). In the case of Sec1, with endogenous SBP2, the double SECIS mutants reduced readthrough from ~3 to ~1% (Fig. 3*A*, compare *lanes 2* and *4*) thus indicating the same relative amount of Sec incorporation as found for Sec5 (Fig. 3*C*, *white bars*). As expected, the addition of 0.2 pmol of CTSBP2 had no stimulatory effect on readthrough for either Sec1 M1M2 or Sec5 M1M2 (Fig. 3*A*, compare *lanes 4* and *5* to *lanes 9* and *10*). That Sec5 M1M2 retained significantly higher readthrough relative to Sec1 M1M2 indicates that most of the readthrough apparent for Sec5, in the absence of added SBP2, is the result of translation infidelity as opposed to Sec incorporation. Table 1 shows the amount of readthrough due to Sec incorporation for Sec1 and Sec5 calculated as the difference between readthrough observed with wild-type SECIS elements and that observed with the double SECIS mutants. These results reiterate that most of the readthrough obtained for Sec5 in the absence of added SBP2 is the result of translational infidelity. However, this activity is suppressed in the presence of saturating levels of SBP2, suggesting that when Sec incorporation is relatively efficient, it is able to suppress nonspecific readthrough of UGA codons.

TABLE 1

Absolute percentage of Sec incorporation of Sec1 and Sec5 (when translated with 0, 0.2, or 2 pmol of CTSBP2) as calculated by subtracting readthrough of their respective double SECIS mutants (Sec1-Sec1 M1M2 or Sec5-Sec5 M1M2)

Construct	Absolute % of Sec incorporation	±S.E.
Sec1; 0 pmol of CTSBP2	2.19	0.29
Sec5; 0 pmol of CTSBP2	2.34	0.7
Sec1; 0.2 pmol of CTSBP2	9	0.7
Sec5; 0.2 pmol of CTSBP2	12.85	0.79
Sec1; 2 pmol of CTSBP2	19	0.97
Sec5; 2 pmol of CTSBP2	20	0.7

To determine whether the Sel P 3′-UTR plays a role in promoting the ability of Sec5 to allow UGA readthrough, reporter

[3] N. Rodriguez and P. R. Copeland, unpublished results.

RNAs lacking a 3′-UTR were synthesized and translated in the presence of endogenous SBP2 or 0.2 pmol of CTSBP2. Fig. 3*B* shows that in the absence of a 3′-UTR, readthrough for both Sec1 and Sec5 is reduced to ~1% in the presence of endogenous SBP2 (Fig. 3*B*, *left panel*, compare *lanes 2* and *3* to *5* and *6*) and even further reduced in the presence of 0.2 pmol of CTSBP2 (Fig. 3*B*, *right panel*, compare *lanes 8* and *9* to *11* and *12*). These data are shown graphically in Fig. 3*C* (*white* and *gray bars*). Together these results suggest that an element within the Sel P 3′-UTR is required for promoting SECIS core-independent UGA readthrough.

To analyze the relative contribution of each SECIS element to readthrough and Sec incorporation, all combinations of SECIS elements with core mutations were created (see Fig. 1) and translated in the presence of endogenous SBP2, and 0.2 and 2 pmol of CTSBP2. The results are shown as a graph of at least three independent experiments (Fig. 3*D*). When the first SECIS element (SECIS 1) of Sec1 was mutated (Sec1 M1), average readthrough dropped by ~50% with both endogenous SBP2 and 0.2 pmol of CTSBP2 (Fig. 3*D*, *white* and *gray bars*). The same mutation in SECIS 2 of Sec1 left readthrough unaltered when translated with 0.2 pmol of CTSBP2. When translated with 2 pmol of CTSBP2, average readthrough of Sec1 M2 was restored to within 5% of wild-type Sec1 (Fig. 3*D*, *black bars*). However, saturating amounts of SBP2 could not increase average readthrough of Sec1 M1 to greater than 4.6% (Fig. 3*D*).

Mutating the core of SECIS 1 in Sec5 had only a slight effect on readthrough in the presence of endogenous SBP2, consistent with the fact that most of the readthrough in this case is due to translation infidelity (Fig. 3*D*, *white bars*). In the presence of 0.2 and 2 pmol of CTSBP2, Sec5 M1 showed ~7 and ~12% average readthrough representing a ~50% reduction when compared with Sec5 with a wild-type SECIS element (Fig. 3*D*, *gray* and *black bars*). A mutation of SECIS 2 in Sec5 caused only a slight reduction in readthrough that was only detectable when translated with added CTSBP2.

Mutating Overlapping UGAs to UGUs Does Not Increase Sec Incorporation to Levels Seen with Sec1–Sec6—Full-length Sel P is produced in cells (12) and we have previously shown that even *in vitro*, despite its numerous UGAs, full-length Sel P can be synthesized, albeit inefficiently (22). Despite the ability of full-length Sel P to be processively translated *in vivo* and *in vitro*, when translated with 0.2 pmol of CTSBP2, Sec7–Sec10 had low average Sec incorporation efficiencies of ~4% (Fig. 2*C*, *gray bars*). To study the effects of individual Sec codon contexts from this region, the overlapping UGAs (Fig. 1, *underlined UGAs*) in Sec7–Sec10 were mutated to the UGU cysteine codon generating the Sec UGU constructs. In reactions containing endogenous SBP2, Sec7–Sec9 UGU provided no detectable readthrough, just as in the case of Sec7–Sec10 containing tandem UGA codons. Sec10 UGU was the exception with barely detectable readthrough (Fig. 4*A*, *white bars*). With 0.2 pmol of CTSBP2, average readthrough of Sec7 UGU and Sec9 UGU was also similar to that for Sec7 and Sec9, remaining unchanged at ~4%, but Sec8 UGU and Sec10 UGU showed a modest increase in readthrough of ~1.5% each (Fig. 4*A*, *gray bars*).

Readthrough of almost all Sec UGU constructs increased 3-fold when translated with saturating amounts of SBP2. However, readthrough levels were about 1.5-fold less than readthrough of Sec1-Sec6 with saturating amounts of SBP2 (compare Fig. 2*C* to Fig. 4*A*, *black bars*). Interestingly, Sec7–Sec10 had strong predicted termination contexts with A residues at every fourth base position. Because we found that a C at the fourth base formed the weakest context for termination (Sec4–Sec6), we mutated the fourth base of the Sec7 UGU mutant from an A to a C. This mutation slightly enhanced readthrough in the presence of endogenous SBP2 and 0.2 pmol of CTSBP2, but in the presence of 2 pmol of CTSBP2, average readthrough decreased slightly to ~10% (Fig. 4*A*). Overall this data indicates that for the codon contexts that require a high level of processivity (*i.e.* closely spaced Sec codons like Sec7–Sec10), the efficiency as measured by this *in vitro* system is markedly lower than that of the other codon contexts, suggesting that there may be another *trans*-factor or *cis*-element specifically required for processive Sec incorporation in Sel P that targets the Sec7–Sec10 codon contexts.

Mutations at the Fourth Base Reduce Sec Incorporation—For a better understanding of the role of codon context

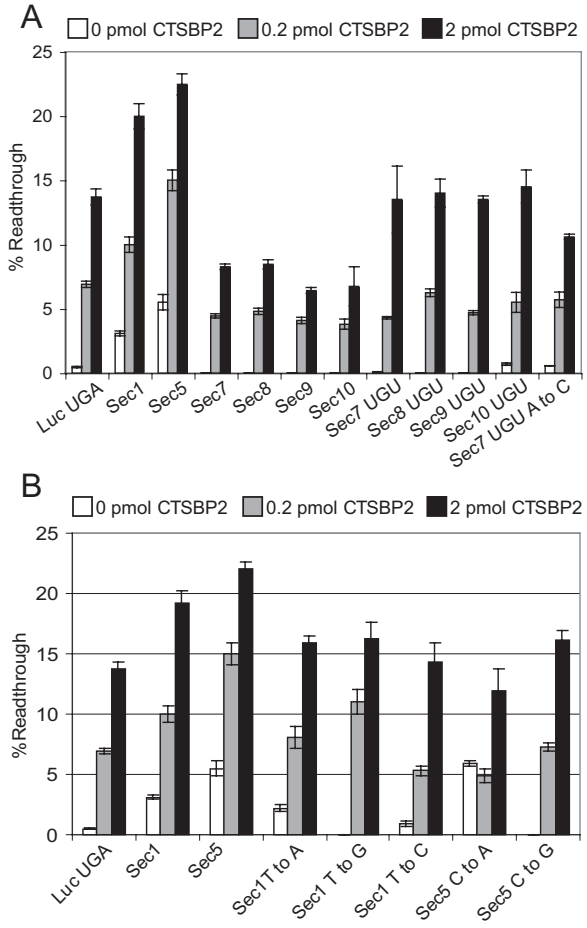

FIGURE 4. **Graphs representing *in vitro* translation of Sec UGU and fourth base mutants.** *A*, after mutating the overlapping UGA in Sec7–Sec10 to UGU, readthrough was studied with endogenous SBP2 (*white bars*), 0.2 (*gray bars*) and 2 pmol (*black bars*) of CTSBP2. The Sec7 UGU fourth base point mutant (A mutated to C) was also translated with and without added SBP2. *B*, fourth base point mutants for Sec1 and Sec5 were translated in the presence of endogenous SBP2 (*white bars*), 0.2 pmol (*gray bars*), and 2 pmol (*black bars*) of CTSBP2. All experiments are shown as the mean ± S.E. of at least three experiments.

and the fourth base on Sec incorporation in rat Sel P, we set out to determine whether the differences in readthrough observed for Sec1 *versus* Sec5 lay in the identity of their fourth base. To systematically evaluate the contribution of the fourth base in readthrough efficiency, we mutated the fourth base of Sec1 from a U to a C, an A, or a G residue and the fourth base of Sec5 from a C to an A or a G (replacement with a U residue was not possible because it would create a UAG stop codon). None of the changes made resulted in the introduction of rare codons that might affect the levels of readthrough by a different mechanism. Regardless of the base, compared with the wild-type context, we saw a decrease in full-length peptide production when the fourth base was mutated (Fig. 4*B*) suggesting that the fourth base may cooperate with another context element in regulating readthrough efficiency. The only exception to this observation was the Sec5 C to A fourth base mutant translated with endogenous SBP2 (Fig. 4*B*, *white bars*). A detailed study of the differences in readthrough of the Sec1 fourth base mutants in the presence of endogenous SBP2 and 0.2 and 2 pmol of CTSBP2 revealed patterns that changed with changes in the concentration of SBP2 (Fig. 4*B*). With endogenous SBP2, Sec1 followed the order of G > C > A > U with G being the most efficient terminator. In this case, the wild-type base, a U, was the least efficient terminator with an average readthrough of about 3% (Fig. 4*B*, *white bars*). With 0.2 pmol of CTSBP2, changing the fourth base of Sec1 to a C decreased readthrough by ~50%, thus changing the hierarchy of bases for termination to C > A > U = G. When translated in saturating amounts of SBP2, the order of termination strengths of the fourth bases were similar to what was seen with intermediate amounts of SBP2 (Fig. 4*B*, *black bars*). Thus, even when the Sec1 context was changed to have the same fourth base as Sec5, readthrough efficiency remained low.

For Sec5 with altered fourth bases, the efficiency of termination followed the order of G > C > A, with an A residue resulting in the most readthrough. In the presence of 0.2 pmol of CTSBP2, an A and a G at the fourth base in Sec5 gave an average readthrough of ~5% (Fig. 4*B*, *gray bars*). Like Sec1, Sec5 followed the same hierarchy of bases for translation termination in the presence of 0.2 and 2 pmol of CTSBP2, yet unlike Sec1, with added SBP2, Sec5 followed the A > G > C rule for translation termination (Fig. 4*B*, *gray* and *black bars*). Overall, these results show that the wild-type fourth base always yielded maximum readthrough, making a strong case for the co-evolution of a fourth base context with other elements within the larger Sec codon context.

Fourth Base Analysis among Different Organisms—For a better understanding of how the fourth base functioned in Sec incorporation, we analyzed the frequency of the 12 nucleotides around the conserved Sec codons of Sel P in rat, mouse, human, bovine, and orangutan (Fig. 5*A*).

In the organisms we chose for our alignment, the 12 nucleotides surrounding the first Sec codon are 96% conserved and with a 91% similarity, the codon context of Sec10 is the next most conserved. Contexts 4 and 5 shared 81% conservation, whereas contexts 6, 8, 3, 9, 7, and 2 were 80, 79, 78, 77, 76, and 67% conserved, respectively (Fig. 5*A*).

A prominent characteristic of Sec4-6, the contexts with the highest Sec incorporation, is that they have a C and an A as their fourth and fifth bases, respectively, and a C or a U immediately 5′ of their Sec codon. Yet, as shown in Fig. 4, changing the fourth base of Sec1 to a C, thus creating a UGACA context, did not increase readthrough significantly. An analysis of all human stop codons using the Transterm data base revealed 31% of them had a G as their fourth base, 29% an A, 21% a C, and 18% a U (25). Two trends are notable from this analysis: 1) the apparent preference for a C at the fourth base and 2) selection against a U in this position. The data presented above as well as previously published work support the preference of a C in the fourth base, but it is clear that the fourth base may play a much less significant role in regulating Sec incorporation than it does in promoting translation termination. This further supports our findings that the fourth base is not a predictor of readthrough efficiency, but it is noteworthy that the human selenoproteome does not adhere to the A > G > C > U rule for termination context strengths.

Sec Incorporation Inhibits Aminoglycoside-induced Translation Readthrough—Aminoglycosides, such as G418, promote translational infidelity by suppressing stop codons (19). To address whether G418 could promote translational infidelity in a context-dependent manner, we *in vitro* translated Luc UGA, Sec1, and Sec5 with G418 ranging from 0 to 24 μg/ml in the absence of added SBP2. G418 has previously been shown to promote UGA and UAG-specific readthrough in RRL at these concentrations (19). At concentrations above 3 μg/ml, overall translation was significantly reduced (Fig. 6*A*, *lanes 6–8*), likely due to G418 toxicity. The maximum efficiency of UGA readthrough occurred in the presence 12 μg/ml at 20% for Luc UGA, and in the presence of 3 μg/ml at 40% for Sec1, and 52% for Sec5 (Fig. 6, *A* and *B*). To examine the interplay between Sec incorporation and G418-induced UGA readthrough we further studied the translation of Sec5 in the presence and absence of added SBP2. [^{35}S]Met incorporation was used to determine total UGA readthrough and ^{75}Se incorporation was used to determine the amount of Sec incorporation. Selenium labeling was achieved by adding [^{75}Se]sodium selenite (0.045 μCi) to a final concentration of 64 nM. This amount of selenium was determined to be the minimum concentration required for maximal Sec incorporation (data not shown). The ^{75}Se-labeled translated product produced a single band that corresponded to the full-length peptide seen with [^{35}S]Met-labeled reactions. The ^{75}Se-labeled product was produced in an SBP2-dependent and SECIS core-dependent manner (data not shown). When Sec5 was translated in the presence of low concentrations of G418 (0, 20, 40, and 80 ng/ml), [^{35}S]Met incorporation into full-length luciferase increased to a maximum of ~15% (Fig. 7, *A*, *odd numbered lanes*, and *B*, *gray bars*). Interestingly, these concentrations were unable to support SBP2-independent Sec incorporation as shown by the lack of ^{75}Se incorporation even at the highest G418 concentration (Fig. 7*C*, *odd numbered lanes*). This was also observed to be the case when we tested a full range of G418 concentrations up to 24 μg/ml (data not shown). In the presence of saturating levels of SBP2, G418 had only a modest effect on total UGA readthrough (Fig. 7, *A*, *even numbered lanes*, and *B*, *black bars*), but it resulted in an ~2-fold reduction in ^{75}Se incorporation.

At a concentration of 20 ng/ml, G418 induced total readthrough to ~6% in the absence of added SBP2, but it did

FIGURE 5. **An alignment of Sec codons and their contexts.** *A*, nucleic acid and alignments of Sel P UGA codons and their contexts (12 flanking bases either side of the Sec codon) in mouse, rat, human, cattle, and orangutan. The Sec codon is shown in *light gray*. *B*, nucleic acid alignments of all human Sel P, rat Sel P, mammalian Sel P, and human Sec codons (shown in *light gray*) and the 12 nucleotides on either side of the Sec codon. Alignments were created using WebLogo (29).

not significantly increase readthrough in the presence of 2 pmol of CTSBP2, suggesting that the process of Sec incorporation inhibits G418-induced translation infidelity.

Sec Incorporation Competes with Translation Termination—As selenocysteine is encoded by a stop codon, it is possible that SBP2 and other components of the Sec incorporation machin-

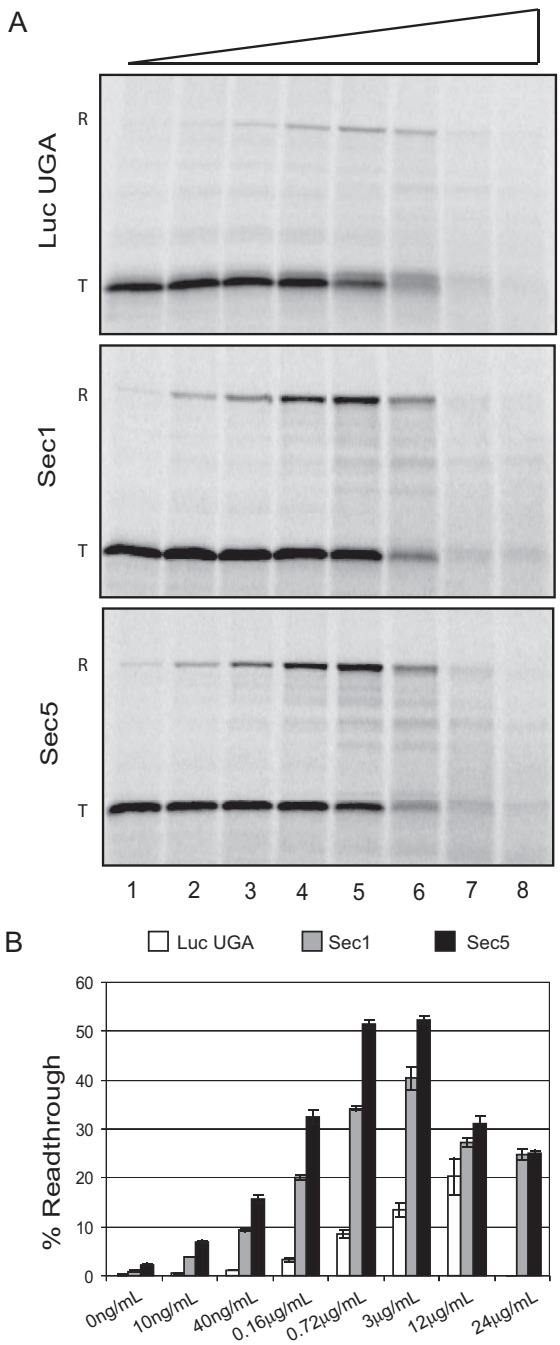

FIGURE 6. ***In vitro* translation of Luc UGA, Sec1, and Sec5 with G418.** *A*, Luc UGA, Sec1, and Sec5, all with wild-type SECIS elements were *in vitro* translated with a range of G418 (0 to 24 ng/ml) in the absence of added SBP2. Gels shown are representative of three independent experiments. *B*, graphical representation of data in *A*. All experiments were performed three times and are shown as ± S.E.

ery compete directly with eukaryotic release factors for access to the UGA codon. To test the effects of eRF1 on Sec incorporation *in vitro*, 25 ng of human eRF1 mRNA (which yielded ~3.5 fmol of translated product) was co-translated with either Sec1 or Sec5 in the presence of endogenous SBP2 or 0.2 pmol of CTSBP2. With added eRF1 and endogenous SBP2, average readthrough of both Sec1 and Sec5 decreased by ~50% (Fig. 8*A*, *lanes 2* and *3 versus 5* and *6*; and *C*). This inhibitory effect of eRF1 was not seen when Sec1 and Sec5 were translated with intermediate amounts of SBP2 (Fig. 8, *B* and *D*). This shows that the ability of SBP2 to stimulate Sec incorporation is not impeded by an increase in eRF1.

That translation termination can occur at a Sec codon is evident by the ample production of pre-Sec peptide in comparison to full-length peptide. The finding that eRF1 lowers readthrough of Sec1 and Sec5 by almost 50% is seen when eRF1 mRNA is co-translated with either of these constructs. However, when RRL is supplemented with just 0.2 pmol of CTSBP2, excess eRF1 is no longer able to terminate as efficiently at the Sec codon. Thus, excess eRF1 is able to inhibit the nonspecific readthrough obtained for Sec1 and Sec5 with endogenous SBP2, but not when SBP2 levels are increased and Sec incorporation efficiency is enhanced. Overall, these data indicate that SBP2 along with the entire Sec incorporation complex directly compete with eRF1 for A-site access.

DISCUSSION

Through *in vitro* translation, mutagenesis, and sequence alignments, we have shown that the conventional rules for weak and strong translation termination contexts do not apply to UGA recoding to Sec in Sel P. The data in Fig. 2 show that the conventional rules for weak and strong termination contexts do not directly determine readthrough efficiency in Sel P and that SBP2 can overcome inherent differences in the translation of different contexts. The most convincing data in support of a larger codon context rather than only the fourth base's involvement in readthrough is that despite a U at the fourth base, Sec1 is not the most efficiently translated construct and that readthrough of Sec3, at subsaturating levels of SBP2, is much less than readthrough of Sec4–Sec6 even though all of these constructs have the same fourth base. A comprehensive mutational analysis of the fourth base of Sec1 and Sec5 revealed that both contexts followed different termination patterns based on the concentration of SBP2 in the reaction. We also learned that although the fourth base did not accurately predict efficiency of translation, with added SBP2, the native context gave the greatest readthrough for both Sec1 and Sec5 (Fig. 4*B*). Consistent with these findings, a thorough alignment of various Sel P Sec codons and their contexts as well as an alignment of the human selenoproteome further supports the observation that Sec codons do not adhere to the conventional rules for translation termination.

Ours is not the first study to show that Sec incorporation may represent an exception to the fourth base rule for translation termination. An earlier study used transiently transfected HEK cells to examine Sec incorporation efficiency. Using various combinations of nucleotides at the fourth and fifth base positions, the authors of that study determined a pyrimidine at the fourth base was a better dictator of termination than a purine at the same position, suggesting that Sec incorporation may deviate from the fourth base rule (21), and that a larger codon context may play a role in determining the efficiency of Sec incorporation. We believe that the codons surrounding the Sec codon may form a *cis*-element and this element dictates readthrough efficiency. The presence of a *cis*-element near the Sec codon would coincide with a recent finding in selenopro-

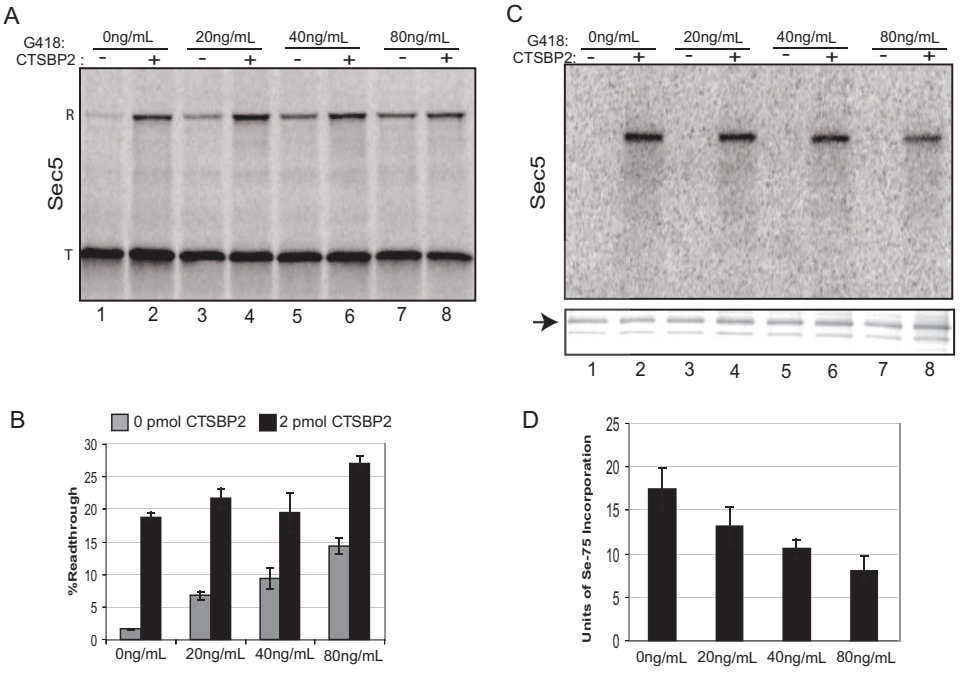

FIGURE 7. ***In vitro* translation of Sec5 with G418 and 2 pmol of CTSBP2.** *A*, representative gel of Sec5 with a wild-type SECIS element *in vitro* translated with either 0 pmol of CTSBP2 (represented as −) or 2 pmol of CTSBP2 (represented as +) and in 0 ng, 20 ng/ml, 40 ng/ml, or 80 ng/ml of G418. The full-length products correspond to readthrough as a result of UGA suppression by G418, whereas the smaller pre-sec peptide is from termination at the UGA codon. *B*, graphical representation of the data in *A*. *C*, *top panel*, Sec5 with a wild-type SECIS element translated with ^{75}Se and 0 pmol (shown as −) or 2 pmol (shown as +) of CTSBP2 and 0 ng, 20 ng/ml, 40 ng/ml, or 80 ng/ml of G418. The only visible product in the *top panel* is the ^{75}Se-labeled full-length peptide. *Bottom panel*, code blue-stained gel of the ^{75}Se-labeled reactions. *Arrow* indicates the band that was used to normalize for loading. *D*, normalized data of ^{75}Se incorporation. All experiments were carried out three times and are shown as an average ± S.E.

tein N, which, in addition to the SECIS element, has a *cis*-element in its coding region that stimulates Sec incorporation (26). Juxtaposing the previous findings to those in this paper, it appears likely that in selenoproteins, a larger codon context in conjunction with the base downstream of the UGA codon, determine readthrough. It is apparent, however, that the sequences that drive efficient readthrough (*i.e.* Sec4–Sec6) do not contain a conserved motif that may explain their commonality (see Fig. 5*A*). In addition, secondary structure predictions of the rat Sel P Sec codon contexts did not reveal a correlation between their free energy and translation (data not shown). We believe the efficiency with which a Sec codon is decoded depends on a larger codon context and concomitantly on the fourth base.

To delineate the contribution and function of each Sel P SECIS element and 3′-UTR in Sec incorporation, we *in vitro* translated Sec1 and Sec5 with single or double SECIS core mutations and Sec1 and Sec5 lacking a 3′-UTR. We found SECIS 1 to be a dominant regulator of Sec incorporation with SECIS 2 seeming largely dispensable. Overall, these results are congruous with the finding from zebrafish Sel P where mutating SECIS 1 was more detrimental to Sec incorporation than mutating SECIS 2 but the latter was required for processive Sec incorporation (13).

Several of the results suggest that the Sec incorporation process requires a modification of the ribosomal A-site. It is interesting to note that the amount of readthrough obtained from Sec5 M1M2 in the presence of 0.2 pmol of CTSBP2 is about 35% lower than that obtained in the presence of endogenous SBP2. Although not statistically significant, this trend suggests that SBP2 may affect readthrough in a SECIS-independent fashion, perhaps as a result of its ribosome binding activity (27). In the absence of a UTR and with endogenous SBP2, readthrough for Sec1 and Sec5 dropped to ~1% and dropped further still with 10 ng of CTSBP2. Although we cannot rule out the possibility that these results may reflect altered reporter mRNA stability, it may be that the level of SBP2 in a given cell type may regulate the ratio of Sec incorporation *versus* readthrough, which may be stimulated under conditions of low SBP2 as a means of preventing rapid mRNA decay via nonsense mediated decay.

The reduced readthrough efficiency observed for the dual UGA constructs indicates that rabbit reticulocyte lysate may be limiting for a factor required for processive Sec incorporation. However, even upon mutating the overlapping UGAs in Sec7–Sec10 to create single-UGA constructs, we were not able to increase readthrough to the same levels as Sec1–Sec6, suggesting that these codon contexts may contain signals for processive Sec incorporation.

To further examine the mechanistic interplay between Sec incorporation and translation termination, we used the aminoglycoside G418 to induce translational infidelity. Interestingly, G418 did induce infidelity in a context-dependent manner and saturating amounts of SBP2 inhibited this infidelity. The fact that Sec incorporation and G418-induced readthrough are not additive provides evidence that the Sec incorporation process regulates A-site access, possibly through SBP2-dependent regulation of conformation. This is consistent with recent results that demonstrated a reduction in G418-induced readthrough of a wild-type but not mutant SECIS element-containing reporter construct (28). That the Sec-tRNA$^{[Ser]Sec}$ does not appear to be among the tRNAs that are allowed access to the A-site during G418-induced readthrough indicates that a step prior to the recognition of cognate *versus* non-cognate tRNA is regulated during Sec incorporation. The amount of readthrough and the difference between Luc UGA and Sec1/Sec5 shown in Fig. 6 is consistent with previous observations in reticulocyte lysate where the fourth base was shown to have a dramatic effect on UGA readthrough (19). The difference between Sec1 and Sec5, however, is likely dictated by a larger context and thus may be more directly related to Sec incorporation. Finally, taking into account the ability of intermediate amounts of SBP2 to suppress the effects of eRF1 (Fig. 8), we believe that the Sec incorporation machin-

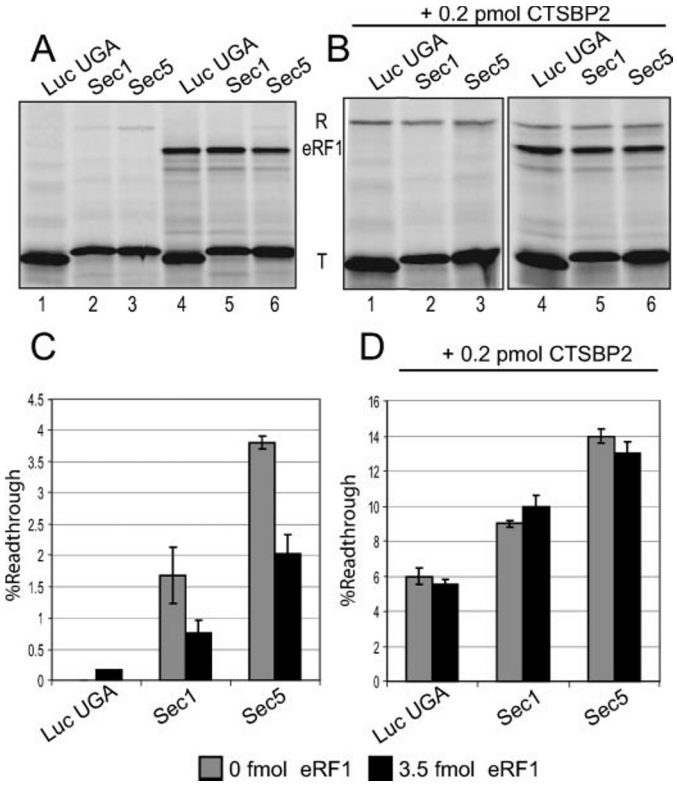

FIGURE 8. ***In vitro* translation in the presence of eRF1.** *A*, Luc UGA, Sec1, and Sec5 were co-translated in endogenous SBP2 with either 0 or 25 ng of eRF1 mRNA. *B*, Luc UGA, Sec1, and Sec5 were co-translated in 0.2 pmol of CTSBP2 with either 0 or 25 ng of eRF1 mRNA. Full-length eRF1 is indicated as are the polypeptides resulting from Luc UGA, Sec1, or Sec5 UGA readthrough (*R*). Polypeptides resulting from termination at the UGA of Luc UGA, Sec1, or Sec5 are also labeled (*T*). *C*, graphical representation of the data in *A*. *Gray bars* represent translation carried out with endogenous SBP2 and 0 ng of eRF mRNA and *black bars* represent constructs co-translated with 25 ng of eRF1 mRNA (3.5 fmol of *in vitro* translated product) and endogenous SBP2. *D*, graphical representation of the data in *B*. All translations were with 0.2 pmol of CTSBP2 and either 0 ng of eRF1 mRNA (*gray bars*) or 25 ng of eRF1 mRNA (*black bars*). Data are represented as the mean ± S.E. of three independent experiments.

ery is in direct competition for the ribosomal A-site and that accommodation of the Sec-tRNA$^{[Ser]Sec}$-eEFSec-GTP ternary complex is distinct from its eEF1A counterpart as well as eRF1.

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