Procesive incorporation of multiple selenocysteine residues is driven by a novel feature of the selenocysteine insertion sequence

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RNA stem loop structures have been frequently shown to regulate essential cellular processes. The selenocysteine insertion sequence (SECIS) element, found in the 3’ UTRs of all selenoprotein mRNAs, is an example of such a structure, as it is required for the incorporation of the 21st amino acid, selenocysteine (Sec). Selenoprotein synthesis poses a mechanistic challenge because Sec is incorporated during translation in response to a stop codon (UGA). Although it is known that a SECIS-binding protein (SBP2) is required for Sec insertion, the mechanism of action remains elusive. Additional complexity is present in the synthesis of selenoprotein P (SELENOP), which is the only selenoprotein that contains multiple UGA codons and possesses two SECIS elements in its 3’ UTR. Thus, full-length SELENOP synthesis requires processive Sec incorporation. Using zebrafish Selenop, in vitro translation assays, and 75Se labeling in HEK293 cells, we found here that processive Sec incorporation an intrinsic property of the SECIS elements. Specifically, we identified critical features of SECIS elements that are required for processive Sec incorporation. A screen of the human SECIS elements revealed that most of these elements support processive Sec incorporation in vitro; however, we also found that the processivity of Sec incorporation into Selenop in cells is tightly regulated. We propose a model for processive Sec incorporation that involves differential recruitment of SECIS-binding proteins.

To date, several RNA secondary structures have been shown to influence essential biological functions via interactions with their cognate RNA binding proteins. One such example is the eukaryotic selenocysteine insertion sequence (SECIS), a conserved stem–loop structure residing in the 3’ UTR of all selenoprotein mRNAs and an essential element for selenoprotein synthesis (1). Selenoprotein synthesis is unique, as it requires co-translational incorporation of the 21st amino acid, selenocysteine (Sec) in response to an in-frame stop codon (UGA). For this purpose, eukaryotic selenoprotein synthesis requires the recruitment of an additional set of translation factors, including a specialized elongation factor (eEFSec), the selenocysteine tRNA (Sec-tRNASe), the SECIS element, and SECIS binding protein 2 (SBP2) (2–5). Together, these factors are sufficient for Sec incorporation in vitro, albeit with lower efficiency (6–8).

Initial attempts to identify the structure of a SECIS element were based on 2D modeling of conserved sequences and site-directed mutagenesis (9–11). Structural probing by enzymatic cleavage and chemical modifications identified a triplet of non-Watson–Crick base pairs with an invariant G-A tandem in the SECIS core. This type of motif was later classified as a kink-turn (K-turn), which is also found in some small nuclear RNAs and rRNA (12). As shown in Fig. 1, SECIS elements exhibit a conserved K-turn motif in the internal loop, made up of GA:GA base pairs (shown in red), which is recognized by its cognate binding protein, SBP2 (5, 13, 14). SBP2 has a canonical L7Ae RNA binding domain that is an established kink-turn binding motif (15). Consensus SECIS elements also carry a stretch of conserved adenines (or, very rarely, cytosines) in the apical loop (9, 10). The high degree of conservation in these features has allowed the development of computational tools that can predict selenoproteins and SECIS elements (16, 17).

Although the interactions between various SECIS elements and SBP2 have been probed, the exact mechanism by which SECIS specifies recoding of an UGA codon for selenocysteine insertion remains elusive. Deeper mechanistic questions arise in the case of SELENOP, which is the only selenoprotein with multiple selenocysteine codons in its primary coding sequence (10–17 depending on the species) and two SECIS elements in its 3’ UTR. Early on, it was speculated that two SECIS elements may be required to recode multiple UGA codons. However, further analysis of the SELENOP 3’ UTR showed that deletion of SECIS-1 resulted in complete loss of processive Sec incorporation in favor of early termination at the second UGA, whereas deletion of SECIS-2 had little or no impact on SELENOP production (18, 19). A similar study in mice showed the same result in terms of processivity, but overall production of SELENOP was reduced for the SECIS-2 deletion (20). Given that both...
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**Figure 1. Schematic of SECIS elements.** Red represents the known conserved motifs of SECIS elements. Each SECIS element is divided into unique sections that include the lower stem, internal loop, upper stem, and apical loop.

SELENOP SECIS elements contain the conserved core motifs and are capable of Sec incorporation, we sought to determine which features within a SECIS element are required for processive Sec incorporation. We found that, although the conserved SECIS core motifs are essential, they are not sufficient for multiple UGA codon redefinitions. We observed that a non-conserved portion of the upper stem and the apical loop of SECIS-1 are necessary and sufficient for processive Sec incorporation. Additionally, our screening of all human SECIS elements revealed four distinct classes of processivity functions. We also observed that \([\text{Mg}^{2+}]\) simulates processivity of SECIS elements in vitro.

**Results**

**SECIS-1 is processive and SECIS-2 is nonprocessive both in vitro and in cultured cells**

To determine the functions of SECIS-1 and SECIS-2 in isolation, we cloned the 119-nt region corresponding to each SECIS element behind the full-length zebrafish Selenop coding region (Fig. 2A). We used the zebrafish version of Selenop because it contains 17 Sec codons and is thus ideally suited for \(^{75}\text{Se}\) labeling in processivity studies (21, 22). For analysis, we employed both in vitro translation assays and transfection into human embryonic kidney cells (HEK293). For in vitro translation, capped mRNAs encoding the SELENOP constructs (Fig. 2A) were translated in rabbit reticulocyte lysate in the presence of \(^{75}\text{Se}\) selenite (Fig. 2B). For expression in cells, SELENOP complementary DNAs were transfected into HEK293 cells, and SELENOP, which is secreted into the medium, was monitored using \(^{75}\text{Se}\) selenite labeling. As shown in Fig. 2B, both the full-length 3’ UTR and SECIS-1 alone yielded robust expression of the full-length product at \(~40\) kDa both in vitro and in cells. However, SECIS-2 alone produced a product that corresponded to termination at the second codon, indicating a complete lack of processive Sec incorporation. Because the first UGA codon has been implicated previously in negatively regulating processivity (19), we also analyzed SELENOP synthesis from the SECIS-2 construct when the first Sec codon was mutated to Cys (U59C). Interestingly, the U59C mutant showed a subtle increase in processivity but was unable to reach the efficiency or level of processivity observed with the full-length 3’ UTR or SECIS-1 alone, both in in vitro translation and in cultured cells. These results indicate that SECIS-1 is uniquely capable of processive Sec incorporation.

The upper stem and apical loop of SECIS-1 are essential for processive Sec incorporation

To identify the determinants of processive Sec incorporation within SECIS-1, we swapped sections of SECIS-1 and SECIS-2 to identify a sequence that is both necessary and sufficient to support processivity. As illustrated in Fig. 1, we divided SECIS into four domains: lower stem, central core, upper stem, and apical loop. To test for sequences that were necessary for processivity, we substituted regions of SECIS-1 for those in SECIS-2, as shown in Fig. 3A. As above, in vitro transcribed capped mRNAs were translated in rabbit reticulocyte lysate in the presence of \(^{75}\text{Se}\) selenite. Replacement of either the upper stem or apical loop of SECIS-1 resulted in complete loss of processivity (Fig. 3A, lanes 6 and 7). To test for sufficiency, we similarly created four SECIS-2 mutants, as shown in Fig. 3B, and placed each of the SECIS-1 sections into a SECIS-2 background. In this case, we were unable to restore processivity with any of the SECIS-1 sections alone. To test the combined action of the SECIS-1 upper stem and apical loop, we created a chimera that contained the lower half of SECIS-2 and the upper half of SECIS-1. Here we observed that processivity was restored to some extent in vitro and fully in transfected cells (Fig. 3C). For all mutant SECIS elements, we used the publicly available computational method (SECISSearch3) for identification of SECIS elements to ensure that they folded into predicted SECIS structures (17). The results show that the upper stem and apical loop of SECIS-1 are necessary and sufficient for processive Sec incorporation both in vitro and in live cells.

A G-rich motif in SECIS-1 is important for SECIS-1 function in processivity

Considering that both the upper stem and apical loop are required for optimal processivity, to identify a requirement for specific sequences, we generated two mutations where these regions converge in the “neck” of SECIS-1. As shown in Fig. 4A, we created a mutation that altered the six bases at the top of the
upper stem as well as the 3’ base of the upper loop. This resulted in changing a G-rich region to an AU-rich region. We also changed an adenosine in the A30-G47 non-Watson–Crick base pair in the upper stem, creating a C-G pair. Both in vitro translation and expression in HEK293 cells, as described earlier, demonstrate that the larger G-rich region is essential for SECIS-1 function in processivity but does not affect overall Sec incorporation activity because the termination product at the second UGA is still detectable, albeit to a lesser extent in cells (Fig. 4B). The overlapping A30C mutation, however, had no impact on processive Sec incorporation. These results allow us to map this novel function for SECIS elements in promoting processive Sec incorporation to the neck region of SELENOP SECIS-1.

**Human SECIS elements are varied in their ability to support processivity**

Because human cells are readily able to make full-length zebrafish Selenop, we reasoned that human SECIS elements would also be able to drive processive Sec incorporation in fish Selenop. Therefore, we tested each of the 26 known human SECIS elements by appending them to the zebrafish Selenop coding sequence. Each of these constructs were tested in vitro and in transfected cells (Fig. 5, A and B, respectively). Both experimental conditions revealed four distinct classes of SECIS element function: "full" processivity (similar to the WT; Fig. 5B, lanes 1 and 2); intermediate processivity (about 50% termination and 50% processivity); no processivity (only production of product terminated at the second UGA); and no Sec incorporation at all. Strikingly, most SECIS elements are processive in vitro, whereas in cells, processivity is restricted to selective SECIS elements. For instance Gpx4, TR1, Gpx6, SPS2, SELENOV, and SELENOM show intermediate processivity in vitro, whereas they are nonprocessive in cells. To ensure that the failure to produce any product in the cells was not due to differential RNA stability, we performed qRT-PCR. As shown in Fig. 5C, the RNA levels of these constructs were similar to or higher than that of the WT construct. These results suggest that processivity is not unique to SELENOP SECIS elements and is more common than expected; however, in cells, this process is more tightly regulated.

**Magnesium stimulates the processivity of partially processive SECIS elements**

Prior studies have confirmed that RNAs containing the K-turn motif are polymorphic and exist in an equilibrium
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Figure 3. Analysis of SECIS-1/SECIS-2 chimeras. A, top panel, schematic of SECIS-1 chimeras wherein either the lower stem, internal loop, upper stem, or apical loop of SECIS-1 is replaced with its equivalent from SECIS-2. SECIS 1 elements are shown in green and SECIS 2 in coral pink. Center panel, capped Zselenop and chimera mRNAs were translated in RRL supplemented with 8 pmol of recombinant CT-SBP2 and analyzed using $^{75}$Se labeling. Bottom panel, $^{75}$Se-labeled medium from transient cell lines expressing the constructs in the top panel, resolved by SDS-PAGE and detected by PhosphorImager analysis. Arrows indicate full-length and early termination products. B, top panel, schematic of SECIS-2 chimeras wherein either the lower stem, internal loop, upper stem, or apical loop of SECIS-2 is replaced with its equivalent from SECIS-1. SECIS 1 elements are shown in green and SECIS 2 in coral pink. Center panel, capped Zselenop and chimera mRNAs were translated in RRL supplemented with 8 pmol of recombinant CT-SBP2 and analyzed using $^{75}$Se labeling. Bottom panel, $^{75}$Se-labeled medium from transient cell lines expressing the constructs in the top panel, resolved by SDS-PAGE and detected by PhosphorImager analysis. Arrows indicate full-length and early termination products. C, top panel, schematic of the processive SECIS-2 chimera containing the upper stem and apical loop of SECIS 1. SECIS 1 elements are shown in green and SECIS 2 in coral pink. Center panel, capped Zselenop and chimera mRNAs were translated in RRL supplemented with 16 pmol of recombinant CT-SBP2 and analyzed using $^{75}$Se labeling. Arrows indicate full-length Zselenop and early termination products. Radiolabeled proteins were resolved by SDS-PAGE and detected by PhosphorImager analysis. Bottom panel, $^{75}$Se-labeled medium from transient cell lines expressing the constructs in the top panel, resolved by SDS-PAGE and detected by PhosphorImager analysis. The arrows indicate full-length and early termination products.

between the strongly kinked conformation and the less-kinked bulge-like structure (23). Depending on the binding of the metal ions, the equilibrium becomes more biased to the highly kinked conformation. To analyze whether the ability of the SECIS to drive processivity is affected by the conformation of the K-turn in the SECIS elements, we selectively tested five SECIS elements for their ability to drive processive Sec incorporation when supplemented with increasing $[\text{Mg}^{2+}]$ concentrations: human SECIS-1 (HSECIS-1), HSECIS2, HDio2 ZSECIS-1, and ZSECIS-2. To determine whether we could restore dominant processivity to either completely nonprocessive (ZSECIS-2 or HSECIS-2) or partially processive (HDio2) SECIS elements, we preincubated in vitro transcribed capped mRNA constructs in the presence of a range of $[\text{Mg}^{2+}]$ prior to translation. For this purpose, we used the Flexi rabbit reticulocyte lysate (nuclease-treated) because this lysate has a known concentration of magnesium and monitored Zselenop synthesis in the presence of $^{[75}\text{Se}]$selenite.

We found that $[\text{Mg}^{2+}]$ concentration influences the processivity of SECIS elements. As shown in Fig. 6, A and B, full-length Zselenop production was not affected by $[\text{Mg}^{2+}]$ concentration when driven by the WT full-length 3’ UTR or ZSECIS1. In contrast, there was a positive correlation between increasing $[\text{Mg}^{2+}]$ and the amount of full-length Zselenop produced for the HSECIS1. Similarly, HDio2 showed increased processivity at higher $[\text{Mg}^{2+}]$ (Fig. 6D). Surprisingly, completely nonprocessive ZSECIS2 yielded a small amount of full-length Zselenop at 1.5–2.0 mM $[\text{Mg}^{2+}]$ even though ZSECIS2 alone has not been reported previously to show processive Sec incorporation (Fig. 6E). On the other hand, HSECIS2 was resistant to processivity at any $[\text{Mg}^{2+}]$ concentration tested.

We also observed that increasing $[\text{Mg}^{2+}]$ concentration not only increased processivity but also increased the efficiency of Sec incorporation at the first UGA. To test whether this result was due to an increase in overall canonical translation, we analyzed WT luciferase with increasing $[\text{Mg}^{2+}]$. We found an over-
all increase in canonical protein synthesis; however, the WT zebrafish 3′ UTR, HSECIS1, and HDio2 showed statistically significant increases above canonical translation at 2.0 mM and 2.5 mM Mg²⁺ (Fig. S2).

Together, these results suggest that the structural conformations of the SECIS elements play a critical role in determining processivity for SECIS elements in vitro. However, currently, because of inadequate structural studies of SECIS elements, we were unable to identify any structural commonalities between the SECIS elements of the four categories identified in Fig. 5.

**Discussion**

SECIS elements have been shown to regulate Sec incorporation both in prokaryotes and eukaryotes. Although SECIS elements in prokaryotes are located immediately downstream of the UGA codon in the coding region, SECIS elements in eukaryotes are more defined and are found in the 3′ UTR (24). Several factors influence the efficiency of Sec incorporation by SECIS, such as the UGA codon position, distance between the UGA and the SECIS core, the SECIS type, the upper stem length and apical loop size in SECIS, and, as discovered very recently, the coding region sequence (25–35). However, the influence of SECIS elements on processive Sec incorporation has never been investigated. Several attempts to identify determinants of processive Sec incorporation have been unsuccessful (18, 19, 36, 37). In this study, we focus on SELENOP SECIS elements 1 and 2 and analyze their differences in driving processive Sec incorporation.

*The upper stem and apical loop of SECIS-1 are essential for SECIS-1 processivity*

Earlier studies by Stoytcheva et al. (19) proposed that Sec incorporation at the first UGA codon is selectively performed...
by SECIS-2. However, we found that, in our in vitro studies (18), SECIS-1 was sufficient to yield a full-length product, whereas SECIS-2 was only capable of Sec incorporation at the first UGA. As shown in Fig. 1, both SELENOP SECIS elements possess identical conserved features known to be required for Sec incorporation; one drives processive Sec incorporation and the
other does not. In the WT SELENOP 3’ UTR, SECIS-2 is located an additional >300 nt downstream of SECIS-1 (Fig. 2A). To test whether the distance between UGA codons and the SECIS core is a determinant of processivity (38), we created SECIS-1 alone and SECIS-2 alone mutants (Fig. 2A) in which the distance between the last UGA and the SECIS core was 77 nt. The optimal spacing recommended between the last UGA and the SECIS core for efficiency is >50 nt (38). However, SECIS-2 alone still caused termination at the second UGA, suggesting that distance was not a determinant. Interestingly, the SECIS-2 U59C mutant showed a subtle stimulation of processivity but still did not yield a full-length protein. On the other hand, the SECIS-1 alone mutant produced a full-length protein similar in efficiency to the WT 3’ UTR but also produced some intermediate products that correspond to termination between the second UGA and the last (17th) UGA. The detection of early termination products by SECIS-1 alone supports earlier studies indicating that although SECIS-2 is not essential for Sec incorporation, it primarily affects decoding at the first UGA (19, 39). Thus, slow decoding at the first UGA by SECIS-2 may be essential to ensure full-length protein synthesis. Upon deeper analysis for the determinants of processivity, we found that the upper stem and the apical loop of SECIS-1 are unique for processivity. In fact, this region was sufficient to make SECIS-2 processive, although at lower efficiency in vitro. Surprisingly, in cultured cells, the SECIS-2 chimera was not only efficient but, like the SECIS-1 alone mutant, produced both full-length protein and early termination products. This suggests that although the upper stem and apical loop of SECIS-1 are critical for processivity, their SECIS-2 counterparts probably regulate decoding efficiency at the first UGA.

In prior studies, sequence analysis of various SECIS elements revealed that the upper stem length is typically restrained to 9–11 bp, or approximately one helical turn of an RNA helix (24). It has been speculated that the optimal upper stem length may serve to juxtapose the conserved motifs in the SECIS core and the adenosine triad in the apical loop in the kink-turn SECIS (24). The reported apical loop size range is typically 10–14 nt (24). In ZSelenop, both SECIS elements constitute a similar upper stem length and apical loop size, which are in the expected range; this fails to explain their difference in function. Sequence analysis of SELENOP SECIS-1 elements among vertebrates revealed a conserved G-rich motif at the junction of the SECIS-1 upper stem and apical loop, which, in this study, seems crucial for processive Sec incorporation. It does not impact single Sec incorporation and processive Sec incorporation may occur as independent events. We also speculated that the predicted non-Watson–Crick bp in the SECIS-1 upper stem (AG) may exist as an extra helical base and be important for processive Sec incorporation. Surprisingly, the AG→CG mutant had no effect on processivity.

**Processivity may be governed both by structure and the ribonucleoprotein complex it recruits**

Our screen of the human SECIS library revealed that most SECIS elements were capable of at least partial processive Sec incorporation in vitro. What we did not expect was the lack of primary sequence similarity among the processive human SELENOP SECIS elements (not even the G-rich motif) other than the already known core motifs among processive SECIS elements. This suggests either a role for RNA secondary structure in regulating processivity or differences in interactions with SECIS binding proteins. Our results imply a role for magnesium in regulating processivity. Magnesium is known to influence K-turn motifs into highly kinked conformation. This may mediate tertiary interactions within the RNA that may either allow or stabilize binding of specific factors in vitro. This correlates well with the lack of sequence conservation between processive SECIS elements outside of the core-binding motif (40).

Biochemical probing of SECIS elements alone (human and rat DIO1 and rat Gpx1) as well as chemical probing of SBP2 in complex with different SECIS elements (rat Gpx1, HuSEL-ENON, rat 5’DIO1, rat Gpx4, rat SELENOP, and mouse SLENOF) show similar cleavage patterns in the SECIS core region. However, clear discrepancies can be seen in the upper stem even though they are all predicted to exist in a double helix (9–11, 14). It is therefore possible that although SBP2 interaction with the core motif and lower stem is constant among all SECIS elements, interactions with additional SECIS binding proteins with the upper stem and apical loop may dictate processivity.

Together, we propose that although processive Sec incorporation can be driven by magnesium concentration in vitro, the mechanism may be different in cells. In cells, SECIS elements differentially drive processive Sec incorporation, which likely depends on the assembly of unique ribonucleoprotein complexes besides SBP2.

**Conclusion**

SECIS elements have long been found essential for Sec incorporation. However, the exact mechanism by which they interact with the rest of the factors is poorly understood. This study provides new insight into regions within the SECIS element that is required for processive Sec incorporation and the influence of processivity. It is therefore possible that although SBP2 interaction with the core motif and lower stem is constant among all SECIS elements, interactions with additional SECIS binding proteins with the upper stem and apical loop may dictate processivity.

Together, we propose that although processive Sec incorporation can be driven by magnesium concentration in vitro, the mechanism may be different in cells. In cells, SECIS elements differentially drive processive Sec incorporation, which likely depends on the assembly of unique ribonucleoprotein complexes besides SBP2.

**Figure 5. Screen for human SECIS elements for processivity.** A, schematic of the ZSelenop coding region ligated to one of the 26 human SECIS elements. B, left panels, capped ZSelenop mRNAs ligated to different human SECIS elements were translated in RRL supplemented with 8 pmol of recombinant CT-SBP2 and analyzed using 75Se labeling. ZSelenop FL3’ UTR, ZSECIS-1, and ZSECIS-2 were used as controls. Radiolabeled proteins were resolved by SDS-PAGE and detected by PhosphorImager analysis. **Right panel,** 75Se-labeled medium from transient cell lines expressing constructs with the ZSelenop coding region ligated to any one of the 26 human SECIS elements, resolved by SDS-PAGE and detected by PhosphorImager analysis. Arrows indicate full-length (FL) ZSelenop and early termination products (Term). C, qRT-PCR analysis of mRNA data obtained from the samples in B that either produced no product or very weak products. Equal amounts of total RNA were used for qRT-PCR, and the -fold increase in coding region deletion mutant mRNA relative to ZSelenop was determined by the comparative C_{\text{T}} method. Data are plotted as the average ± S.D. for three independent experiments. p < 0.05 compared with the native Zselenop value.
ence of magnesium on SECIS function. Thus, it serves as a foundation for future research exploring the differential decoding efficiency and processivity of SECIS elements, which we speculate occurs by recruiting additional RNP complexes besides SBP2.

Materials and methods

Constructs

The ZSelenop plasmid in the pcl-Neo vector was a gift from Dr. Vadim Gladyshev (Harvard Medical School, Boston, MA).
This construct was FLAG-tagged using the QuickChange site-directed mutagenesis kit (Agilent) according to the manufacturer's instructions, and the resulting product was TA-cloned into a mammalian expression vector, pcDNA3.1, using the TOPO-TA cloning kit (Invitrogen). pcDNA3.1 also contains the G418 resistance gene. The Pac1 site was created after the stop codon using the QuickChange site-directed mutagenesis kit (Agilent) according to the manufacturer's instructions. The pcDNA3.1 vector has a natural NotI site. To create SECIS-1- or SECIS-2-only mutants, the SECIS element, as predicted using SecISsearch along with an additional 20 nt 5' and 3' of the predicted SECIS, was PCR-amplified using Pac1 and NotI primers and then ligated in-context to the native ZSelenop coding region.

**SECIS elements and mutants**

The SECIS-1 and SECIS-2 mutants (mut 1A-1D and mut 2A-2D), the SECIS 2 chimera, the G-rich AU mutant, and the AG-to-CG mutant were synthesized commercially from Integrated DNA Technologies. To screen the SECIS library, SECIS elements were amplified using Pac1 and NotI linkers and then ligated downstream of the ZSelenop coding region.

**Cell culture and transfection**

Untransfected HEK293 cells cultured in Lonza Eagle's Minimum Essential Medium (with Earle's Balanced Salt Solution and L-glutamine), containing 10% fetal bovine serum, maintained at 37 °C with a humidified 5% CO2 atmosphere were used for transient transfection of the WT and mutants of ZSelenop SECIS elements with 10-fold lower amounts of GFP to monitor transfection efficiencies. For transfection, jetPRIME (Polyplus) reagent was used according to the manufacturer’s protocol. 5–6 × 103 cells/well were seeded 24 h prior to transfection in a 6-well plate. 24 h post-transfection, the medium was changed to serum-free EMEM that was supplemented with 100 nM 75Se (specific activity, 6.29 Ci/mM). For analysis by 12% SDS-PAGE, the adhered cells were then gently washed with cold PBS and lysed in 1% NP-40 buffer (50 mM Tris–HCl (pH 8.0), 150 mM sodium chloride, 1% NP-40, and Roche cOmplete protease inhibitor). The lysate was then cleared by centrifugation at 17,000 × g for 10 min at 4 °C. The precleared lysate was analyzed by SDS-PAGE to ensure similar transfection efficiencies.

**In vitro RRL translation**

The in vitro translation assay was performed as described earlier (18). In vitro transcribed and capped mRNA of either SELENOP WT or mutants were translated in rabbit reticulocyte lysate nuclease–treated (Rabbit Reticulocyte Lysate System, Nuclease Treated, L4960) or Flexi rabbit reticulocyte lysate (Flexi® Rabbit Reticulocyte Lysate System, L4540) supplemented with 8 pmol of CT-SBP2 and 75Se. Translation reactions were incubated for 1 h at 30 °C. 4 µl of the translation products was then resolved by 12% SDS-PAGE gel and quantitated by PhosphorImager analysis (GE Healthcare).

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