jmb Communication

Reconstitution of Selenocysteine Incorporation Reveals Intrinsic Regulation by SECIS Elements

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http://dx.doi.org/10.1016/j.jmb.2013.04.016
Edited by R. L. Gonzalez

Abstract

Selenoproteins are present in all three domains of life and are responsible for a major part of a cell's antioxidant defense against reactive oxygen species. Synthesis of selenoproteins requires the decoding of a UGA codon as selenocysteine (Sec) instead of translation termination. Sec is incorporated into the growing polypeptide chain during translation elongation and is known to require a set of highly specific factors: the Sec insertion sequence (SECIS) element in the 3′ untranslated region, Sec-tRNA^{Sec}, the Sec-specific elongation factor eEFSec, and SECIS binding protein 2. Since reconstitution has not been reported, whether these factors are sufficient is unknown. Here, we report a novel *in vitro* translation system in which Sec incorporation has been reconstituted from purified components introduced into a Sec naive system. In addition, we developed a novel method to purify Sec-tRNA^{Sec} and active eEFSec/GTP/tRNA ternary complex. We found that the known basal factors are sufficient for Sec incorporation *in vitro*. Using this highly manipulable system, we have also found that ribosomes from non-Sec-utilizing organisms cannot support Sec incorporation and that some SECIS elements are intrinsically less efficient than others. Having identified the essential set of factors, this work removes a significant barrier to our understanding of the mechanism of Sec incorporation.

Introduction

Selenium is cotranslationally incorporated as the 21st amino acid selenocysteine (Sec) into selenoproteins in all three domains of life, although their expression is notably lacking in higher plants and fungi. There are at least 25 selenoproteins in humans, and their functions range from reduction of oxidized phospholipids to protein folding.[1] Deletion of Sec-tRNA^{Sec} is embryonic lethal in mice, thus accentuating the biological importance of Sec incorporation.[2] During translation of selenoprotein mRNAs, Sec is encoded by an in-frame UGA codon, which is normally a stop codon. To date, it is known that this recoding event requires three *trans*-acting factors [Sec-tRNA^{Sec}, Sec-specific elongation factor eEFSec, and the Sec insertion sequence (SECIS) binding protein 2 (SBP2)] and one *cis*-acting RNA sequence [the SECIS element in the 3′ untranslated region (UTR)]. While other factors have been suggested to be involved in the Sec incorporation process, only these four factors have been shown to be required, although they have never been shown to be sufficient.

Eukaryotic SECIS elements are stable stem–loop structures that belong to the kink-turn family of RNA structures. They are required for Sec incorporation and are exclusively found in the 3′ UTR. They are composed of two helices separated by an internal loop of 4–18 nucleotides,[3] and they have two conserved regions: the SECIS core (RUGA, where R = A or G) and an apical AAR motif. The SECIS core forms the base of helix 2, which contains four non-Watson–Crick base pairs, the tandem G.A/A.G "quartet" and a 5′ RU.[4] The core is also the binding site for SBP2, and this binding is essential for Sec incorporation.[5,6] SECIS elements also have an apical AAR motif usually composed of three unpaired adenosines that are required for Sec incorporation,[7] but the function of this AAR motif is still unknown. SECIS-specific regulation of selenoprotein expression has been studied before using all

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26 human SECIS elements.[8] These studies suggested that the SECIS elements differ in their UGA recoding efficiency, but whether the differences were due to intrinsic properties of the SECIS elements or the function of an unknown protein factor was not determined.

SBP2 was first detected as a protein that specifically cross-linked to the Gpx4 3′ UTR with a wild-type SECIS[9] and was subsequently shown to be required for Sec incorporation.[5] SBP2 specifically binds to the SECIS core, and its binding is not influenced by mutations in the conserved AAR motif of the SECIS element.[6,9] It was subsequently shown that mammalian SBP2 consists of three domains: an N-terminal domain that is dispensable for Sec incorporation *in vitro*, a central Sec incorporation domain that is required for Sec incorporation and wild-type levels of SECIS binding, and the C-terminal RNA binding domain, which contains a canonical L7Ae RNA binding domain that is required for SECIS binding.[10,11] Studies of structure–function relationships within SBP2 have been greatly aided by the rabbit reticulocyte lysate (RRL) *in vitro* translation system, which is replete with all Sec incorporation factors except SBP2.

A specialized translation elongation factor, eEFSec, is required for Sec incorporation in eukaryotes

Identification of the eukaryotic Sec-specific translation elongation factor by homology to EF-Tu, eEF1A, and archaeal SelB was reported independently by two groups.[12,13] eEFSec is a G-protein that binds GTP and GDP with similar affinity, and thus it likely does not require guanine exchange factor.[12,13] The same studies have also demonstrated that eEFSec specifically binds Sec-tRNA^{Sec} but not its precursor, Ser-tRNA^{Sec}. eEFSec has four domains, and based on sequence conservation, the first three domains of eEFSec are similar to the canonical eukaryotic translation elongation factor eEF1A, but it has a C-terminal extension termed Domain IV. Recently, it has been shown that this domain is required for Sec incorporation and is involved in Sec-tRNA^{Sec} binding, GTPase regulation, and interactions with SBP2 in a SECIS-dependent manner.[14] This study employed a partially reconstituted *in vitro* translation system that was limiting for eEFSec, thus allowing the study of eEFSec-dependent Sec incorporation to show that Domain IV is required for all of the known functions for eEFSec: Sec-tRNA^{Sec} binding, GTP hydrolysis, and Sec incorporation.[14]

Mechanistically, it has been presumed that SBP2 and the SECIS element provide the specificity needed for decoding only select in-frame UGA codons by the eEFSec ternary complex, thus preventing translation termination. Since Sec incorporation has not been reconstituted *in vitro*, the exact mechanism of this specificity still remains unclear. In this study, we have created a novel *in vitro* translation system in which Sec incorporation was reconstituted from purified components added to a Sec-naive wheat germ *in vitro* translation lysate. Using this system, we report three major findings: (1) the known core factors are sufficient for Sec incorporation, (2) ribosomes from non-Sec-utilizing species cannot support Sec incorporation, and (3) SECIS elements possess intrinsically different capacities to promote Sec incorporation *in vitro*.

Results and Discussion

A functional assay to determine minimum requirements for Sec incorporation

One of the major hurdles in determining the core mechanism of Sec incorporation is creating a system in which Sec incorporation can be reconstituted from purified factors. A relatively small set of *trans*- and *cis*-acting factors is known to be required for Sec incorporation, but whether these factors are sufficient is unknown. Higher plants do not utilize Sec and they do not possess any of the factors required for selenoprotein synthesis.[15] They are, therefore, a potentially useful system to study reconstitution and modification of the Sec incorporation pathway. In an attempt to reconstitute Sec incorporation *in vitro*, we used wheat germ lysate as a source of translation factors and energy required to support the reaction. To this lysate, we added purified recombinant Sec incorporation factors including the fully active Xpress-His tagged C-terminal fragment of SBP2 (XH-CTSBP2), FLAG tagged eEFSec (FLAG-eEFSec), total aminoacyl tRNA (aa-tRNA) from rat testis (which is a rich source of Sec-tRNA^{Sec}), and mammalian ribosomes. Sec incorporation was monitored with a luciferase reporter consisting of the luciferase coding region with a single in-frame UGA codon at position 258 followed by the rat Gpx4 SECIS element (Fig. 1a). This assay is a sensitive measure of Sec incorporation since the production of full-length and active luciferase is strictly SECIS and SBP2 dependent as demonstrated by the lack of luciferase activity when mutant SECIS elements lacking the conserved AUGA motif are used.[16] As shown in Fig. 1b (lane 1), the addition of the Sec incorporation factors described above was sufficient to observe luciferase activity that was about 6-fold higher than the background obtained from the mRNA with a mutant SECIS element. Figure 1b also shows that reactions lacking any one or all of the components were devoid of Sec incorporation activity (lanes 2–6). These data clearly show that the known factors are sufficient for Sec incorporation *in vitro*. To further verify that the incorporation event

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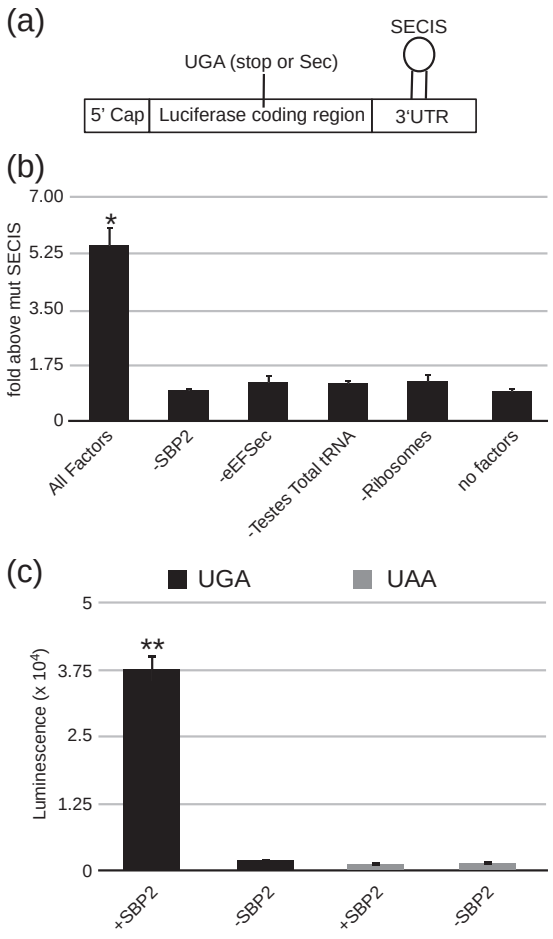

Fig. 1. Reconstitution of Sec incorporation in wheat germ *in vitro* translation lysate. *In vitro* translation of the Sec incorporation reporter mRNA (a) in 50% of wheat germ lysate in the presence or absence of 160 nM XH-SBP2 and FLAG-eEFSec recombinant proteins, 1.25 μg of total testes aminoacylated tRNA, and 80 nM salt-washed rabbit ribosomes (b). Data were normalized for luciferase activity from mutant Gpx4 SECIS element. The data represent the mean and standard deviation of three independent experiments ($n = 3$). The asterisk (*) denotes a significant difference *versus* no factors by Student's *t* test ($p < 0.02$). (c) *In vitro* translation of a Sec incorporation reporter mRNA that has a UAA codon instead of the UGA codon shown in (a). Raw luciferase activity (luminescence) was measured by luminometry. The double asterisk (**) denotes a significant difference *versus* no SBP2 by Student's *t* test ($p < 0.02$).

was selenocysteine, we tested a luciferase construct possessing a UAA stop codon at position 258. In this case, no luciferase activity above the control with a mutant SECIS element was observed (Fig. 1c). Considering that an unknown factor may be carried in our ribosome preparation, we gradient purified ribosomal subunits and found a similar level of activity (~6-fold above background), making it unlikely that the ribosomes are a source of an unidentified essential factor (data not shown). It is clear from previous studies that there is a complex interaction between *trans*- and *cis*-acting factors during Sec incorporation. This novel assay can be used to study the function and dynamics of each of the factors involved and it will also enable the screening for *cis*- and *trans*-acting enhancers or suppressors of Sec incorporation.

Purification of Sec-tRNA^{Sec}

The current method of purification for Sec-tRNA^{Sec} is labor intensive and requires the use of three different columns, one of which is not commercially available.[17,18] This makes Sec-tRNA^{Sec} a limiting reagent in the field of selenium biology, and there is thus a need for a simple and fast method for purification of Sec-tRNA^{Sec}. Since it has been previously shown that eEFSec specifically binds Sec-tRNA^{Sec},[12,13] we decided to perform affinity purification using recombinant FLAG-eEFSec, which should selectively bind Sec-tRNA^{Sec} from a pool of total aa-tRNAs extracted from rat testis. For this, anti-FLAG magnetic beads were incubated with FLAG-eEFSec for an hour, following which total rat testis aa-tRNA and GTP were added and incubated for another hour. Finally, the eEFSec/GTP/tRNA ternary complex was eluted with FLAG peptide and part of the resulting eluate was used directly for a Sec incorporation assay and the other part was used to extract Sec-tRNA^{Sec}. As shown in Fig. 2a, we observed Sec incorporation that was ~9.5-fold above the mutant SECIS background upon addition of the purified Sec-tRNA^{Sec} obtained from the eEFSec pulldown (lane 2) as well as from the eEFSec/GTP/tRNA ternary complex (lane 3). Both of these sources of Sec-tRNA^{Sec} showed greater activity than the addition of total testis aa-tRNA (lane 1). Sec incorporation was not observed in reactions lacking added Sec-tRNA^{Sec} or FLAG-eEFSec (lanes 4 and 5). The presence of Sec-tRNA^{Sec} was confirmed and quantified by Northern blot, and based on this method of detection, we have estimated a purification of approximately 35-fold (Fig. 2b). Together, these results demonstrate that we have developed a method to obtain highly enriched and functional Sec-tRNA^{Sec} and also we have isolated an active eEFSec/GTP/tRNA ternary complex. This is the first time it has been shown that eEFSec, GTP, and Sec-tRNA form an active complex that is able to support Sec incorporation *in vitro*. One of the major mechanistic questions in Sec incorporation is how the delivery of Sec-tRNA^{Sec} by eEFSec to the ribosome is regulated. Since we have established a rapid method to purify Sec-tRNA^{Sec} and an assay to test its functionality, the feasibility of this type of work is greatly increased.

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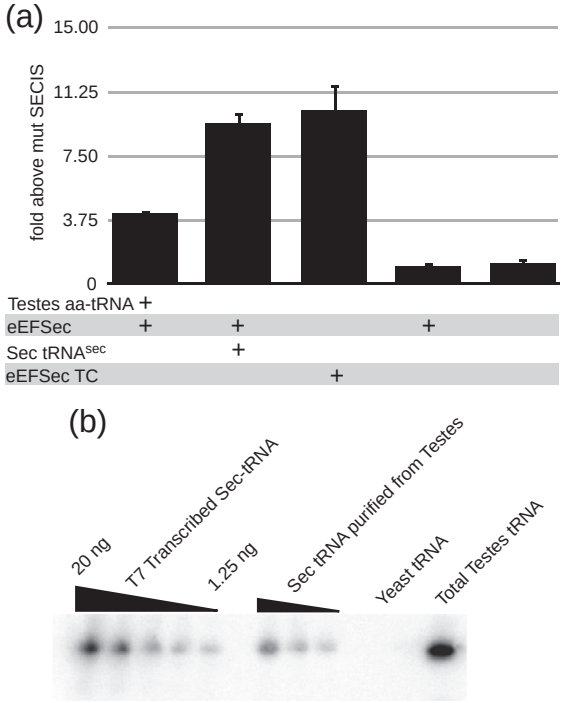

Fig. 2. Purification of *Sec-tRNA*^{Sec}. (a) *In vitro* translation of the Sec incorporation reporter mRNAs as described in Fig. 1 in the presence of total testis aa-tRNA, 125 ng of Sec-tRNA^{Sec}, purified FLAG-eEFSec, and/or purified FLAG-eEFSec/GTP/tRNA ternary complex (TC) as indicated. The data represent the mean and standard deviation of three independent experiments. (b) The presence of Sec-tRNA^{Sec} was confirmed and quantified by Northern blot. Twofold serial dilution of *in vitro* transcribed tRNA^{Sec} (1.25–20 ng) and samples derived from tRNA purifications as indicated were analyzed by Northern analysis and hybridized to a probe complementary to the anticodon loop of tRNA^{Sec}. The amount of Sec-tRNA^{Sec} from each source was determined by densitometry using the *in vitro* transcribed tRNA^{Sec} as a standard curve.

Ribosomes from non-Sec-utilizing organisms are not able to support Sec incorporation

Selenoproteins are present in all three domains of life. However, the distribution of these selenoproteins among eukaryotic taxa varies greatly. For instance, there are no selenoproteins in yeast, higher plants, and several insect species, including the fall armyworm, *Spodoptera frugiperda*. This fact allows us to ask the important question as to whether all ribosomes are intrinsically able to incorporate selenocysteine or whether specific ribosomal structures may have evolved to function in Sec-utilizing organisms. Previous work has shown that SBP2 is able to specifically modify the conformations of helix 89 and expansion segment 31 in mammalian large subunit rRNA.[19] Since neither of these regions is well conserved, it is possible that Sec-specific functionality could reside at these locations. To examine this hypothesis, we tested ribosomes for Sec incorporation as well as total translation from Sec- and non-Sec-utilizing organisms. For this assay, RRL was depleted of ribosomes by centrifugation, and the post-ribosomal supernatant was used to examine the function of exogenous ribosomes in Sec incorporation. Purified salt-washed ribosomes derived from RRL, *S. frugiperda* Sf21 cells, wheat germ lysate, and *Saccharomyces cerevisiae* were added to the ribosome-depleted RRL. Sec incorporation was studied using the luciferase construct described in the previous section. Total translation was measured using a luciferase coding region without any in-frame UGA codon and without a SECIS element. Figure 3 shows total translation (Fig. 3a) and Sec incorporation (Fig. 3b) derived from ribosomes of varying origin. Notably, the ribosomes from non-Sec-utilizing *S.*

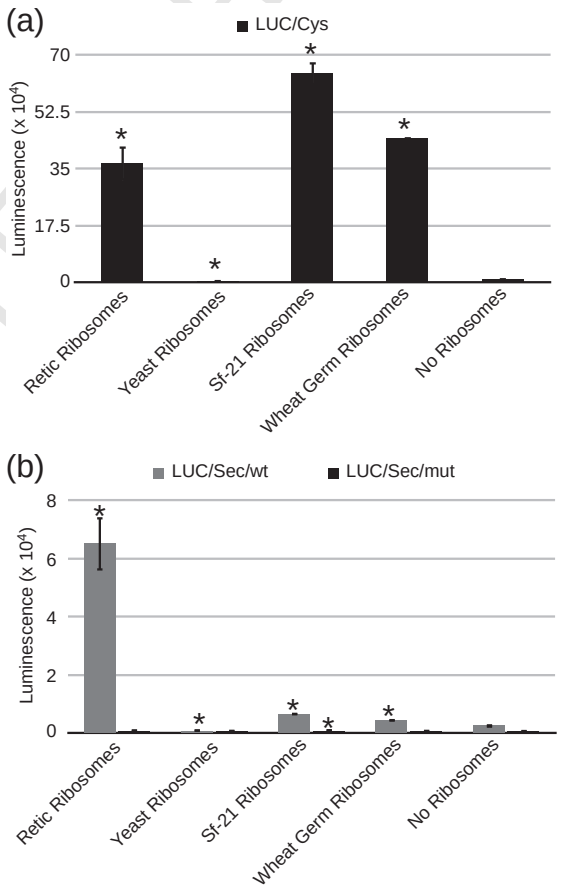

Fig. 3. Mammalian ribosomes are required for Sec incorporation. Total translation (a) or Sec incorporation (b) in ribosome-depleted RRL in the presence of 80 nM salt-washed ribosomes from RRL, *S. cerevisiae*, *S. frugiperda* Sf21 cells, or wheat germ lysate as indicated. The asterisk (*) denotes a significant difference *versus* no ribosomes by Student's *t* test ($p < 0.02$).

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frugiperda and wheat germ can readily support total translation but only very low levels of Sec incorporation. Ribosomes from Sec-utilizing rabbit reticulocyte could support translation as well as Sec incorporation. Interestingly, however, yeast ribosomes could not support total translation or Sec incorporation. The lack of yeast ribosome activity for general translation is a surprise since they have previously been used successfully with mammalian elongation factors.[20] The quality of the yeast ribosomes was verified using a poly(Phe) synthesis assay (data not shown), so it is likely that one or more mammalian initiation factors are incompatible with yeast ribosomes. Although there was a small but statistically significant amount of Sec incorporation with plant and insect ribosomes, the extremely low levels of activity indicate that there is a fundamental difference at the ribosomal level between Sec- and non-Sec-utilizing species. This finding has significant implications for efforts to reconstitute Sec incorporation in genetically tractable Sec-naive organisms such as yeast, but at the same time, it offers a means to potentially screen ribosomal mutant libraries for a gain of Sec incorporation activity, thus potentially permitting an unprecedented level of understanding regarding the molecular mechanism of Sec incorporation.

The role of SECIS elements on expression of selenoproteins

Previously, the UGA recoding efficiency of all human SECIS elements was analyzed *in vitro* using RRL. This study revealed significant differences in recoding efficiency depending on the SECIS element used.[8] Since RRL is only limiting for SBP2 and is replete for all the other Sec incorporation factors,[16] it remains unknown whether the differences in the UGA recoding efficiency can be attributed to an intrinsic property of the SECIS element or an as-yet unidentified factor. Having developed a system free of other Sec incorporation factors, we decided to study the UGA recoding efficiency of three human SECIS elements—glutathione peroxidase 4 (hGpx4), thioredoxin reductase 3 (hTrxR3), and Selenoprotein O (SelO)—using our novel *in vitro* translation system in which Sec incorporation is dependent on externally added Sec incorporation factors. Prior work showed that use of the Gpx4 SECIS element resulted in more than 4-fold greater Sec incorporation efficiency than the TrxR3 SECIS element and more than 50-fold more than the SelO SECIS element in RRL.[8] To test whether this difference is due to an intrinsic property of the SECIS element and its interactions with the known Sec incorporation factors or the ribosome, we used the same luciferase reporter described above but followed by the human Gpx4, TrxR3, or SelO SECIS element. As a positive control, we also tested these constructs in RRL. As shown in Fig. 4, Sec incorporation activity of the hGpx4 SECIS element was found to be about 2-fold higher than that of hTrxR3 and about 30- or 50-fold higher than SelO in wheat germ (Fig. 4a) or RRL (Fig. 4b), respectively. This difference cannot be attributed to differential SBP2 binding since it was previously shown with the same SECIS elements that SBP2 binds TrxR3 (4 nM) and SelO (7 nM) with slightly higher affinity than Gpx4 (13 nM).[21] In addition, the difference observed here is not due to differential RNA stability since equal amounts of the protein product that resulted from termination at the UGA codon were observed when reactions were labeled with ^{35}S methionine (data not shown). We therefore conclude that the difference in the recoding efficiency is an intrinsic property of SECIS elements, likely at the

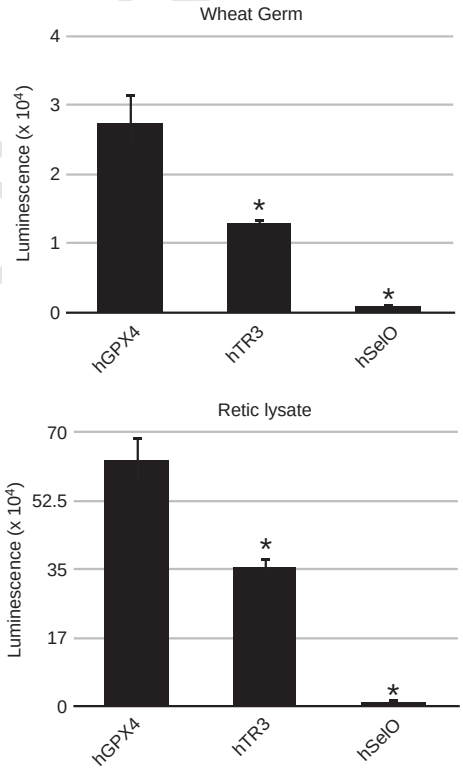

Fig. 4. Differential SECIS element efficiency. (a) *In vitro* translation of the Sec incorporation reporter mRNA bearing the hGpx4, hTrxR3, and SelO SECIS elements in wheat germ lysate with 160 nM XH-SBP2, eEFSec/GTP/tRNA ternary complex, and 80 nM salt-washed rabbit ribosomes. The data represent the mean and standard deviation of three independent experiments. (b) Same as in (a) except the translation was performed in RRL without added ribosomes. The data represent the mean and standard deviation of three independent experiments. The asterisk (*) denotes a significant difference *versus* hGpx4 by Student's *t* test ($p < 0.02$).

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level of direct SECIS–ribosome interactions during decoding.

Conclusion

Here, we have demonstrated that no additional factors are required for the core Sec incorporation reaction. The efficiency of Sec incorporation using this *in vitro* translation assay was found to be 3% of total translation, whereas it has been reported that the efficiency of Sec incorporation using RRL is in between 7% and 10% of total translation. Although this difference is likely due to the inability of the wheat ribosomes to participate in Sec incorporation, it is also possible that there is a factor (or factors) that can be added to increase the efficiency of Sec incorporation. Several factors have been implicated in being involved in Sec incorporation. In one case, ribosomal protein L30 is being supplied as part of our ribosome prep, so we cannot assess its essentiality. Other factors that are not found in wheat, such as SECp43,[22] nucleolin,[23] and the GTPase-activating protein GAPsec,[24] can be tested in this system for specific roles in the core Sec incorporation reaction. Indeed, one of the most important aspects of this work is having established a system in which the individual functions of these and other as-yet undiscovered regulatory factors can be tested in a quantitative and controlled fashion.

Materials and Methods

Recombinant protein expression and purification

Recombinant SBP2 was purified as described previously.[25] FLAG-eEFSec was purified using anti-FLAG M2 magnetic beads (Sigma-Aldrich) as previously described.[14] Quantitation of the recombinant proteins was performed on SDS-PAGE gels using an ovalbumin standard curve.

Extraction of total aminoacylated tRNA from testes extract

Fresh trimmed rat testes were purchased from Pel Freeze (St. Louis, MO). The tissue was homogenized using a hand blender in translation buffer (20 mM Tris–HCl, 100 mM KCl, 2.5 mM $MgCl_2$, 2 mM DTT, 0.4 mM GTP, 0.25 mM spermidine, 20% glycerol, and Roche EDTA-free protease inhibitors). Crude extract was centrifuged at 12,000***g*** for 30 min at 4 °C. Supernatant was collected and used for extraction of aminoacylated tRNA. To 20 ml of the supernatant, 12 ml of H_2O, 8 ml of 5× Buffer T [50 mM NaOAc, 3.25 M NaCl, 50 mM $MgCl_2$, and 5 mM ethylenediaminetetraacetic acid (EDTA)], and 40 ml of phenol, pH 4, was added. The solution was briefly vortexed and centrifuged at 12,000***g*** for 10 min at 4 °C. The aqueous phase was transferred to another tube and re-extracted with 1 volume of phenol, pH 4, to remove remaining protein contamination. RNA was precipitated with 2.5 volumes of 100% ethanol and stored at −80 °C for 10 min. RNA was pelleted at 12,000***g*** for 15 min at 4 °C and resuspended in 1.6 ml of 1× Buffer T. RNA was re-pelleted by ethanol precipitation, washed once with 70% ethanol, and air-dried for 10 min. Pellet was resuspended in H_2O. To verify that aminoacyl tRNA was recovered, we deacylated an aliquot of the purified tRNA in Buffer N (25 mM Tris, pH 9) at 37 °C for 30 min. This deacylated tRNA did not support Sec incorporation or eEFSec binding (data not shown).

In vitro reconstitution of Sec incorporation

Sec incorporation activity was measured with a luciferase mRNA reporter containing a UGA-Sec codon at position 258 of the coding region and the rat Gpx4 SECIS element in the 3′ UTR.[16] Nonspecific read-through activity was measured using a similar luciferase mRNA reporter but the AUGA core (SBP2 binding site) in the SECIS element was deleted. All RNAs have an encoded 100-nt poly(A) tail, which is not required for *in vitro* translation or Sec incorporation,[26] except for those used in Figs. 1c and 4. A typical reaction contained 6.5 μl of wheat germ extract, 100 ng of luciferase mRNA reporter, 160 nM SBP2 and eEFSec recombinant proteins, 1.25 μg of total testes aminoacylated tRNA, and 80 nM salt-washed ribosomes from RRL. Sec incorporation and read-through reactions were incubated at 30 °C for 1 h and measured for luminescence in a 96-well plate luminometer (Berthold Tristar).

Purification of Sec-tRNA^{Sec}

Two liters of bacterial culture over-expressing Flag-eEFSec was pelleted and resuspended in 40 ml of Buffer A (20 mM Tris–HCl, pH 7.5, 20 mM KCl, 0.1 mM EDTA, 25% glycerol, 500 mM NaCl, 1% Tween, and 0.5 mM PMSF). Solutions were sonicated 4 times at 2 s/ml with 1–2 min resting periods on ice and then centrifuged at 15,000***g*** for 15 min at 4 °C. Next, 1 ml of anti-FLAG M2 magnetic beads (Sigma-Aldrich) was incubated with a total of 80 ml of protein extract, in 40-ml aliquots, for 2 h each at 4 °C. After the binding step, the beads were washed 5 times with Buffer A without PMSF followed by 5 times with Buffer B (20 mM Tris–HCl, pH 7.5, 20 mM KCl, 0.1 mM EDTA, and 25% glycerol). Total testes aa-tRNA (7.5 mg) and 0.5 mM GTP were added to the washed beads and incubated at 4 °C for 1 h to obtain pure Sec-tRNA^{Sec}. Protein was eluted in 200 μl of Buffer B with 250 μg/ml of 3× FLAG peptide for 30 min at 4 °C. Half of the eluent was used for Sec incorporation assays and the other half was used for tRNA extraction as described in the previous section. The tRNA obtained after extraction was used for the Sec incorporation assay and Northern analysis.

Northern analysis

Total tRNA extracted from testis, purified Sec-tRNA^{Sec}, and T7 polymerase transcribed Sec-tRNA^{Sec} were loaded onto gels and then electroblotted onto a nylon membrane. The membrane was hybridized with ^{32}P-labeled Sec-tRNA probe using ULTRAhyb-Oligo (Ambicon) solution and conditions. The washed membrane was exposed to a PhosphorImager screen and radioactive signal quantitated using IMAGEQuant software. The probe sequence is CAGCTACAGGTTTGAAGCCTGCACC.

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Purification and assay of ribosomes

80S ribosomes from RRL, wheat germ, and Sf-21 lysate (Promega) were purified as described previously.[19] Purification of 40 and 60S subunits from RRL was performed as reported earlier.[27] To deplete ribosomes, we centrifuged RRL at 300,000***g*** for 1 h at 4 °C. The supernatant was removed and centrifuged again for 1 h at 4 °C. The supernatant following the second centrifugation was used for the assay. Total translation was measured using a wild-type luciferase reporter.[16] Sec incorporation activity and nonspecific read-through activity were measured using the same constructs as described in the previous section. Reactions (12.5 μl) contained 8 μl of ribosome-depleted RRL, 100 ng of luciferase mRNA reporter, 160 nM SBP2, and 80 nM salt-washed ribosomes from RRL. Total translation, Sec incorporation, and read-through reactions were incubated at 30 °C for 1 h and measured for luminescence in a 96-well plate luminometer (Berthold Tristar).

Constructs and in vitro translation to test the role of SECIS elements

The luciferase reporter construct with a UGA codon at position 258 and rat Gpx4 SECIS has been previously described.[16] The human Gpx4, TrxR3, and SelO SECIS constructs were subcloned as PacI/NotI restriction fragment into the luciferase reporter plasmid. The reporter plasmids bearing the human Gpx4, TrxR3, and SelO SECIS elements were linearized with NotI and used as templates to transcribe capped mRNAs with the T7 mMessage/mMachine kit (Ambion) according to the manufacturer's protocol. Both the *in vitro* transcribed mRNAs were used in the Sec incorporation assay described in the previous section as well as in RRL described previously.[16]

Acknowledgements

We thank Arjun Sasikumar for help with ribosomes and critical discussions. This work was supported by Public Health Service grants GM094833 and GM077073 (P.R.C.).

Received 17 January 2013;
Received in revised form 20 March 2013;
Accepted 17 April 2013

Keywords:
ribosome;
translation elongation;
selenocysteine;
SECIS

Abbreviations used:
SECIS, Sec insertion sequence; UTR, untranslated region; SBP2, SECIS binding protein 2; RRL, rabbit reticulocyte lysate; hGpx4, glutathione peroxidase 4; hTrxR3, thioredoxin reductase 3; SelO, Selenoprotein O; EDTA, ethylenediaminetetraacetic acid.

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Please cite this article as: Gupta, N. *et al.*, Reconstitution of Selenocysteine Incorporation Reveals Intrinsic Regulation by SECIS Elements, *J. Mol. Biol.* (2013), http://dx.doi.org/10.1016/j.jmb.2013.04.016

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Please cite this article as: Gupta, N. *et al.*, Reconstitution of Selenocysteine Incorporation Reveals Intrinsic Regulation by SECIS Elements, *J. Mol. Biol.* (2013), http://dx.doi.org/10.1016/j.jmb.2013.04.016