The utilization of selenocysteine-tRNA[Ser]Sec isoforms is regulated in part at the level of translation in vitro

Bradley A. Carlson a, Nirupama Gupta b, Mark H. Pinkerton b, Dolph L. Hatfield a, and Paul R. Copeland a, b

aMolecular Biology of Selenium Section, Mouse Cancer Genetics Program, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; bDepartment of Biochemistry and Molecular Biology, Rutgers - Robert Wood Johnson Medical School, Piscataway, NJ, USA

ABSTRACT

The tRNA for the 21st proteinogenic amino acid, selenocysteine, exists in mammalian cells as 2 isoforms differing by a single 2'-O-methylribosyl moiety at position 34 (Um34). These isoforms contain either 5-methoxycarbonylmethyluridine (mcm5U) or 5-methoxycarbonylmethyl-2'-O-methyluridine (mcm5Um) at position 34. The accumulation of the mcm5Um isoform is tightly correlated with the expression of nonessential "stress response" selenoproteins such as glutathione peroxidase 1 (GPX1). The expression of essential selenoproteins, such as thioredoxin reductase 1 (TXNRD1), is not affected by changes in Sec-tRNA[Ser]Sec isoform accumulation. In this work we used purified mcm5U and mcm5Um Sec-tRNA[Ser]Sec isoforms to analyze possible differences in binding to the selenocysteine-specific elongation factor, EEFSEC, and the translation of GPX1 and TXNRD1 in vitro. Our results indicate that no major distinction between mcm5U and mcm5Um isoforms is made by the translation machinery, but a small consistent increase in GPX1 translation is associated with the mcm5Um isoform. These results implicate fundamental differences in translation efficiency in playing a role in regulating selenoprotein expression as a function of isoform accumulation.

KEYWORDS

glutathione peroxidase; isoforms; methylation; selenium; selenocysteine; thioredoxin reductase; translation; tRNA modification

Introduction

The 21st proteinogenic amino acid, selenocysteine (Sec), is brought to the elongating ribosome as Sec-tRNA[Ser]Sec. Although Sec-tRNA[Ser]Sec is undermodified relative to other tRNAs, it does contain a dynamic methylation in the anticodon loop at uridine 34 (U34), which is synthesized on 5-methoxycarbonylmethyluridine (mcm5U) giving rise to 5-methoxycarbonylmethyl-2'-O-methyluridine (mcm5Um). The synthesis of the mcm5Um isoform of Sec-tRNA[Ser]Sec (hereafter referred to simply as either mcm5U or mcm5Um) depends on the unique tertiary structure of tRNA[Ser]Sec, and its presence has a dramatic impact on the overall structure of the tRNA. It has been recently established at the biochemical and genetic levels that methylcarboxymethylation at position U34 is important for the stable interaction of AAA, CAA and GAA codons in yeast. The additional functionality conferred by the further methylation to mcm5Um has also been linked to translational fidelity, but the mechanism has not been studied.

In mammals, the regulated synthesis of mcm5Um is apparently unique to Sec-tRNA[Ser]Sec, and it is dependent on the presence of an adenosine residue at position 37, prior base modifications at positions 55 (pseudouridine) and 58 (1-methyladenosine), intact secondary and tertiary structures, and aminoacylation of tRNA[Ser]Sec, as well as the prior methylcarboxymethylation at position U34. The isopentenylation of Sec-tRNA[Ser]Sec is performed by the TRIT1 enzyme, genomic deletion of which results in a complete loss of the mcm5Um isoform. The production of mcm5U is catalyzed by the ALKBH8 methyltransferase, which has also been demonstrated to be essential for mcm5Um production in ALKBH8 null mice. The mcm5U and mcm5Um isoforms have been proposed to be differentially used by the nonessential “stress-related” selenoprotein genes such as GPX1. When mcm5Um production is inhibited by mutating the U34 or A37 positions of Sec-tRNA[Ser]Sec, the production of the stress-related selenoproteins, including GPX1, is dramatically reduced. Interestingly, the extent of mcm5Um modification is dependent on the

CONTACT Paul R. Copeland paul.copeland@rutgers.edu Rutgers-Robert Wood Johnson Medical School, 675 Hoes Ln, Piscataway, NJ 08854, USA. © 2017 Taylor & Francis
cellular selenium concentration, thus providing a mechanistic basis for the well known hierarchy of selenoprotein expression during selenium deficiency.\textsuperscript{15} Consistent with these findings, mice lacking TRIT1 activity have reduced expression of the nonessential selenoproteins,\textsuperscript{13} and the ALKBH8 null mice also showed reduced GPX1 levels, albeit to a lesser extent.\textsuperscript{12}

The mechanism by which nonessential selenoprotein expression is regulated by the presence or absence of mcm\textsuperscript{5}Um Sec-tRNA\textsuperscript{[Ser][Sec]} has not been explored. Using GPX1 as an example, one possibility is that the mcm\textsuperscript{5}U isoform is excluded from recruitment to ribosomes loaded with GPX1 mRNA, which might result from differential affinity to the Sec-specific elongation factor EEFSEC. Alternatively, it could be that utilization of the mcm\textsuperscript{5}U isoform for GPX1 translation signals the degradation of GPX1 mRNA in a process akin to nonsense mediated mRNA decay (NMD).\textsuperscript{16-18} This latter hypothesis is supported by the fact that several studies have demonstrated that GPX1 mRNA is targeted for destruction during selenium deficiency,\textsuperscript{19-22} which correlates with the loss of mcm\textsuperscript{5}Um Sec-tRNA\textsuperscript{[Ser][Sec]}\textsuperscript{3,23,24}

In this study, we used the purified Sec-tRNA\textsuperscript{[Ser][Sec]} isoforms to determine whether the mcm\textsuperscript{5}Um isoform is preferentially used during \textit{in vitro} translation and/or elongation factor binding. Although we observed no difference between mcm\textsuperscript{5}U and mcm\textsuperscript{5}Um Sec-tRNA\textsuperscript{[Ser][Sec]} utilization in rabbit reticulocyte lysate, we did observe a small and consistent increase in translation efficiency for GPX1 (nonessential), but not for the essential thioredoxin reductase (TXNRD1) mRNA in a wheat germ system that lacks endogenous Sec-tRNA\textsuperscript{[Ser][Sec]}. However, we also did not observe preferential binding of either of the 2 isoforms by EEFSEC, which is consistent with the idea that the regulation of mcm\textsuperscript{5}Um utilization must occur after this binding event.

\textbf{Results & discussion}

\textbf{Recombinant EEFSEC does not show a preference for binding to either mcm\textsuperscript{5}U or mcm\textsuperscript{5}Um isoforms}

GPX1 protein expression is found to be dramatically reduced \textit{in vivo} when the expression of the mcm\textsuperscript{5}Um isoform is inhibited.\textsuperscript{10,14} One explanation for this result may be that the mcm\textsuperscript{5}U/EEFSEC ternary complex may be intrinsically less stable than the mcm\textsuperscript{5}Um/EEFSEC complex. To determine whether the Sec-tRNA\textsuperscript{[Ser][Sec]} isoforms are differentially bound by the Sec-specific elongation factor, EEFSEC, we took advantage of the well-established method of determining the ratio of mcm\textsuperscript{5}U to mcm\textsuperscript{5}Um by RPC5 chromatography.\textsuperscript{25-27} Fig. 1A shows the characteristic separation of wild-type mouse liver-derived tRNA\textsuperscript{[Ser][Sec]} that was aminoacylated \textit{in vitro} with \textsuperscript{3}H Ser, the early and late-eluting peaks corresponding to the mcm\textsuperscript{5}U and mcm\textsuperscript{5}Um isoforms, respectively. To test EEFSEC binding, we performed affinity purification of Sec-tRNA\textsuperscript{[Ser][Sec]} from mouse liver total RNA with immobilized FLAG tagged recombinant EEFSEC. The EEFSEC-affinity purified tRNA was subject to deacylation and reacylation with \textsuperscript{3}H Ser to verify integrity (Fig. 1B), and the distribution of Sec-tRNA\textsuperscript{[Ser][Sec]} isoforms that were recovered by EEFSEC affinity chromatography was determined by RPC-5 chromatography. Fig. 1C and D show the profiles of Sec-tRNA\textsuperscript{[Ser][Sec]} isoforms derived from total liver mRNA (Fig. 1C) versus that from EEFSEC affinity (Fig. 1D). The fact the EEFSEC-purified and total liver Sec-tRNA\textsuperscript{[Ser][Sec]} profiles are similar indicates that EEFSEC does not preferentially bind to one isoform or the other under these conditions. These results indicate that the strong cellular discrimination between the utilization of mcm\textsuperscript{5}U and mcm\textsuperscript{5}Um isoforms in protein synthesis\textsuperscript{9,10,14} is not likely occurring at the level of EEFSEC binding due to the slight differences we observed in their binding levels to this cellular component. We performed this experiment a single time due to limiting access to equipment and material. However, we are unable to discern a significant bias in EEFSEC binding, and the results observed are well within the typical variance we have found in this assay since it was introduced by us and used for quantifying the levels of the 2 Sec tRNA isoforms under many different physiologic conditions.\textsuperscript{9,10,14,28} In addition, considering this assay used purified components, we cannot rule out the contribution of cellular factors that may regulate EEFSEC affinity.

\textbf{In vitro translation of GPX1 and Selenop mRNAs in rabbit reticulocyte lysate is not dependent on the Sec-tRNA\textsuperscript{[Ser][Sec]} isoform}

Since EEFSEC appears to form a ternary complex with mcm\textsuperscript{5}U just as well as mcm\textsuperscript{5}Um, it is possible that the differential utilization observed in cells
occurs at the level of translation. To test whether there is an intrinsic difference in GPX1 translation as a function of tRNA\textsuperscript{Ser}\textsuperscript{Sec} isoform, we performed \textit{in vitro} translation experiments in rabbit reticulocyte lysate. For this experiment, \textsuperscript{75}Se-labeled mcm\textsuperscript{5}U and mcm\textsuperscript{5}Um isoforms were purified by RPC-5 chromatography. As shown in Fig. 2A, we obtained 2 well resolved peaks, the first corresponding to mcm\textsuperscript{5}U and the latter to mcm\textsuperscript{5}Um.

Early and late fractions from each peak were pooled so as to reduce the chance of isoform mixing. These purified forms of Sec-tRNA\textsuperscript{Ser}\textsuperscript{Sec} were added to rabbit reticulocyte lysate \textit{in vitro} translation reactions containing either GPX1 or SELENOP mRNA. As shown in Fig. 2B, \textsuperscript{75}Se-labeled bands corresponding to the expected molecular weights for both SELENOP and GPX1 were observed. Interestingly, we observed no discernible difference in

---

**Figure 1.** Analysis of Sec-tRNA\textsuperscript{Ser}\textsuperscript{Sec} isoforms by RPC-5 chromatography. (A) Purified \textsuperscript{3H}Ser-tRNA\textsuperscript{Ser}\textsuperscript{Sec} was resolved into the 2 characteristic isoform peaks by RPC-5 column chromatography. Fractions were analyzed by scintillation counting. (B) Sec-tRNA\textsuperscript{Ser}\textsuperscript{Sec} purified by EEFSEC affinity was aminoacylated \itshape in vitro \upshape with \textsuperscript{3H}Ser as described in the experimental procedures. Total liver tRNA (C) or tRNA eluted from anti-FLAG beads loaded with FLAG-EEFSEC (D) was applied to an RPC-5 column and tRNA\textsuperscript{Ser}\textsuperscript{Sec} was detected in fractions by dot blot hybridization with a \textsuperscript{32}P labeled probe.
Figure 2. In vitro translation of GPX1 and SELENOP mRNAs in rabbit reticulocyte lysate using purified $^{75}$Se-labeled Sec-tRNA$^{[Ser]}_{Sec}$. (A) Large scale purification of the mcm$^5$U and mcm$^5$Um isoforms of Sec-tRNA$^{[Ser]}_{Sec}$ by RPC-5 chromatography. The shaded areas represent the fractions that were pooled for each isoform. (B) 100 ng each of GPX1 and SELENOP mRNAs were translated in rabbit reticulocyte lysate in the presence of inorganic $^{75}$Se or the Sec-tRNA$^{[Ser]}_{Sec}$ isoforms as indicated. The fold increase in SELENOP and GPX1 translation as a function of the presence of mcm$^5$Um Sec-tRNA$^{[Sec]}_{Sec}$ is indicated below.
the efficiency of translation for either mRNA as a function of isoform. As a control, reactions were also performed in the presence of free \(^{75}\)Se-selenite which is incorporated into the endogenous tRNA\[^{[\text{Ser}]\text{Sec}}\] in rabbit reticulocyte lysate. We chose to use the SELENOP mRNA because it contains 10 Sec codons, and thus represents a unique challenge to the Sec incorporation machinery. As such, we expected that SELENOP translation might be a sensitive indicator of differences in tRNA isoform utilization, but under these conditions, such a difference was not observed. Overall, these results do not reveal a difference in isoform utilization, but the fact that substantial endogenous Sec-tRNA\[^{[\text{Ser}]\text{Sec}}\] is present in rabbit reticulocyte lysate\(^{29}\) is a complicating factor if pre-formed EEFSEC/Sec-tRNA\[^{[\text{Ser}]\text{Sec}}\] ternary complex is somehow affecting isoform utilization.

**In vitro translation of GPX1 mRNA in a reconstituted Sec incorporation system is slightly enhanced with the \(\text{mcm}^5\text{Um}\) isoform**

Because the rabbit reticulocyte lysate system contains endogenous Sec-tRNA\[^{[\text{Ser}]\text{Sec}}\], we endeavored to use a more defined system to analyze any

---

**Figure 3. In vitro translation of GPX1 and TXNRD1 in wheat germ lysate using purified \(^{75}\)Se-labeled Sec-tRNA\[^{[\text{Ser}]\text{Sec}}\].** (A) A range of \(\text{mcm}^5\text{U}\) and \(\text{mcm}^5\text{Um}\) Sec-tRNA\[^{[\text{Ser}]\text{Sec}}\] amounts from 2000–6000 cpm were added to wheat germ *in vitro* translation reactions programmed with 4 nM of either GPX1 (lanes 1–7) or TXNRD1 mRNA (lanes 8–14). The fold increase in GPX1 and TXNRD1 translation as a function of the presence of \(\text{mcm}^5\text{Um}\) Sec-tRNA\[^{[\text{Ser}]\text{Sec}}\] is indicated below. (B) 1 and 4 nM GPX1 or TXNRD1 mRNAs were translated in rabbit reticulocyte lysate and wheat germ lysate. The fold increase of GPX1 and TXNRD1 in reticulocyte lysate vs. wheat germ lysate is indicated below.
potential difference between the 2 tRNA\(^{\text{SerSec}}\) isoforms. To that end, we used the wheat germ lysate system that we recently developed to characterize the function of EEFSEC.\(^{30}\) Since higher plants do not utilize Sec and do not possess any of the Sec incorporation components, it is an ideal system in which to analyze the core mechanism of Sec incorporation factors. We are unable to use the SELENOP mRNA in this system as the incorporation of multiple Sec residues in a single protein does not occur in these conditions.\(^{31}\) Instead, we generated mRNA encoding mouse TXNRD1, the expression of which is not regulated in vivo by altered Sec-tRNA\(^{\text{SerSec}}\) isoforms.\(^{10,14}\) Based on the cellular and in vivo data, we expected that GPX1 would be translated with higher efficiency with the mcm\(^5\)Um-containing tRNA while TXNRD1 mRNA should be translated with equal efficiency regardless of isoform.\(^{10,14}\) We used a range of tRNA concentrations to examine the dynamic response of GPX1 vs. TXNRD1 translation in wheat germ lysate. As shown in Fig. 3, GPX1 translation appears to be slightly enhanced in the presence of the mcm\(^5\)Um isoform by a maximum of \(\approx\) 2-fold. Interestingly, the overall efficiency of TXNRD1 translation is much higher than that of GPX1 in wheat germ lysate, regardless of isoform. To determine if the difference in efficiency of GPX1 vs. TXNRD1 mRNA translation was specific for the wheat germ system, we used rabbit reticulocyte lysate and wheat germ lysate in the same experiment for a direct comparison. As seen in Fig. 3B, the translation efficiency of GPX1 and TXNRD1 was similar in rabbit reticulocyte lysate but TXNRD1 synthesis was up to 31-fold higher in wheat germ. Considering the difference in TXNRD1 translation efficiency is specific to the wheat germ lysate, we expect this is due to the longer 3' UTR on TXNRD1 mRNA (663 nucleotides) compared with that on GPX1 (181 nucleotides), which is a known factor regulating translation efficiency in the wheat system.\(^{32}\)

**TXNRD1 translation outcompetes that of GPX1**

To further analyze the dynamics of GPX1 vs. TXNRD1 translation, we performed a mixing experiment where the mRNAs were translated either alone or as an equimolar mixture. Interestingly, as shown in Fig. 4, we observed a significant decrease in GPX1 synthesis but the slight enhancement with mcm\(^5\)Um is retained (Fig. 4A, Figure 4. In vitro translation of GPX1 and TXNRD1 together in wheat germ lysate. (A) GPX1 and TXNRD1 mRNAs were translated in the presence of mcm\(^5\)U and mcm\(^5\)Um Sec-tRNA\(^{\text{SerSec}}\) isoforms as described in Figure 3 but in lanes 3 and 6, equal amounts of mRNA were combined in the same reaction. Phosphorimage quantitation of bands plotting the percent increase as a function of using the mcm\(^5\)Um Sec-tRNA\(^{\text{SerSec}}\) isoform is shown below. (B) Phosphorimage quantitation of multiple experiments comparing the amount of GPX1 or TXNRD1 translation in the presence of mcm\(^5\)U vs. mcm\(^5\)Um Sec-tRNA\(^{\text{SerSec}}\). A total of 19 experiments are included in this analysis, which is an unpaired 2-tailed t-test.
compare lanes 3 and 6). This result is consistent with prior work in vivo that the translational efficiency of TXNRD1 appears to be significantly higher than that of GPX1.33,34

To maximize the use of a small supply of purified isoforms, we quantified multiple non-identical experiments in which we compared GPX1 translation vs. TXNRD1 translation in the presence of mcm5Um or mcm5Ua (n = 19). We found an average of 1.7-fold increased GPX1 translation with mcm5Um but little detectable increase (1.1-fold) for TXNRD1. The experiments included in this analysis include all of those shown in Figs. 3 and 4, plus additional experiments that only differed in the amounts of tRNA or mRNA added. This difference was found to be statistically significant in a 2-tailed unpaired t-test (Fig. 4B; p = .002). Whether the small increase in efficiency in the presence of the mcm5Um isoform is functionally significant remains to be tested.

Conclusion

In vivo, the preferential utilization of mcm5Um in stress-related selenoprotein expression has been shown to be stringent, since mice with mutations in either position 34 or 37 of Sec-tRNA[Ser]Sec lack the mcm5Um isoform,2 resulting in a low level of expression for this subclass of selenoproteins.10,14 The purpose of the present study was to elucidate at which step in translation the involvement of mcm5Um occurs in regulating stress-related selenoprotein expression. Our data clearly show that there is no discrimination between mcm5U and mcm5Um at the level of EEFSEC selection, and thus, the distinction must occur at a subsequent step in translation. Removing many of the variables associated with cell and animal based systems, we found that both GPX1 and TXNRD1 can be translated by using either the mcm5U or mcm5Um isoforms in rabbit reticulocyte lysate. However, this observation may indicate that the selectivity observed in vivo for preferential utilization of mcm5Um has been lost in lysate preparation and/or in carrying out translation under these conditions. On the other hand, we do observe a small but reproducible increase in GPX1 translation in the presence of the mcm5Um isoform in wheat germ extract, which may indicate, at least in part, the basis for a cellular mechanism that distinguishes the translation of essential and non-essential selenoprotein mRNAs. This intrinsic difference in efficiency, for example, may be just enough to trigger a response that ultimately leads to GPX1 mRNA decay, thus eliminating GPX1 expression when mcm5Um concentrations are low.

Experimental procedures

Binding of Sec-tRNA[Ser]Sec to EEFSEC

Total tRNA was purified from mouse liver as described previously.26 Sec-tRNA[Ser]Sec was purified by EEFSEC affinity as described previously.30 Briefly, FLAG-EEFSEC was bound to anti-FLAG beads (Sigma-Aldrich) in Buffer B (20 mM Tris-HCl pH 7.5, 20 mM KCl, 0.1 mM EDTA, 25% glycerol) and incubated with mouse liver amino acyl tRNA in the presence of 0.5 mM GTP at 4°C for 1 h. The EEFSEC/Sec-tRNA[Ser]Sec complex was eluted in 200 μl of Buffer B with 250 μg/ml of 3X FLAG peptide for 30 min at 4°C.

Analysis of EEFSEC-bound Sec-tRNA[Ser]Sec

Purified Sec-tRNA[Ser]Sec was deacylated in 1 M Tris (pH 8.0) at 37°C for 1 h. Decacylated tRNA[Ser]Sec was precipitated with 3 volumes of ethanol, collected by centrifugation at 14,000 RPM for 15 min, washed with 75% ethanol, dried and dissolved in nuclease-free H2O. Yeast total tRNA (600 μg) was added to the tRNA[Ser]Sec as a carrier and tRNA was chromatographed on an RPC-5 column in a linear gradient from 0.525 M sodium chloride to 0.675 M sodium chloride containing 10 mM sodium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 4.5.26

Dot blot analysis was performed by spotting 5 μl of each column fraction on a Hybond-N+ membrane (GE Healthcare Life Sciences) followed by UV crosslinking. Hybridization was performed using Quikhyb solution (Stratagene) for 4 h at 58 °C in a rotating hybridization oven with a [32P]-end labeled oligo probe complementary to tRNA[Ser]Sec (5'-CAG ACC ACT GAG GAT CAT CCG-3'), which was prepared using T4 polynucleotide kinase (New England Biolabs) and γ-32P ATP (Specific activity 3000Ci/mmol, Perkin Elmer) according to manufacturer’s instructions. Following hybridization, the membrane was washed 3 times with 2X SSC, 0.1% SDS, then washed 2 times with 0.1X SSC, 0.1% SDS, exposed to a PhosphorImager screen and spots were quantitated using
Aminoacylation of purified tRNA\textsuperscript{[Ser]Sec} was performed using \textsuperscript{[\textit{H}]Ser (specific activity 20Ci/mmol, from Moravek Biochemicals) as described\textsuperscript{26} and labeled tRNA\textsuperscript{[Ser]Sec} was chromatographed on an RPC-5 column as described above.

\textit{Isolation of [\textit{Se}]\textsuperscript{75}-labeled Sec-tRNA\textsuperscript{[Ser]Sec}}

HL-60 cells were grown in the presence of 300 nM sodium selenite as described previously.\textsuperscript{24} Cells (~5 g) were collected, washed with PBS and re-suspended in 75 ml of RPMI-1640 medium containing 1% FBS without sodium selenite. Five millicuries of neutralized \textsuperscript{75}Se (Specific activity ~1000 Ci/mmol, University of Missouri Research Reactor Center, Columbia, MO) were added, and the cells were gently shaken for 3 h at 37°C. Cycloheximide (100 \textmu M final concentration) was then added and cells were incubated for an additional 45 min. Cells were collected by centrifugation at 1,200 RPM for 5 min at 4°C, washed with ice cold PBS, and stored at −80°C until tRNA extraction. Phenol/chloroform extraction and purification of \textsuperscript{75}Se]-labeled Sec-tRNA\textsuperscript{[Ser]Sec} by RPC-5 chromatography was performed as described.\textsuperscript{24}

\textit{GPX1 and TXNRD1 cloning and mRNA synthesis}

Full length mouse GPX1 (coding region plus 181 base pair 3’ UTR) was isolated from mouse liver cDNA by PCR with a Kozak consensus sequence-containing 5’ primer (5’ GCC ACC ATG TGT GCT GCT CGG CTC TCC-3’), and a reverse primer ending immediately upstream of the polyadenylation signal (5’ CTT AGT AGT GAA AC ACC TTT-3’). Partial length mouse TXNRD1 (full coding region plus 663 base pair 3’ UTR) was cloned from mouse cortex cDNA using a Kozak consensus sequence-containing 5’ primer (5’-GCC ACC ATG CCA GTT GAT GAC TGC TGG-3’) and 3’ primer ending 983 base pairs upstream of the polyadenylation signal. Plasmid DNA was linearized with XbaI and 5’ capped mRNA was synthesized by \textit{in vitro} transcription (mMessage, Ambion).

\textit{In vitro translation}

Wheat germ \textit{in vitro} translation reactions (12 \textmu l) contained 6.5 \textmu l of wheat germ extract (Promega), GPX1, SELENOP or TXNRD1 mRNA as indicated in the figure legends, \textsuperscript{[\textit{Se}]\textsuperscript{75}}-labeled tRNA (~6000 cpm unless otherwise indicated) and 320 nM each of C-terminal SECIS binding protein 2 and EEFSEC recombinant proteins as described previously.\textsuperscript{30} Reactions were incubated at 25 °C for 2 hours followed by treatment with 10 \mu g of RNase A for 15 min. at 37 °C. Rabbit reticulocyte lysate (Promega) reactions (12 ul) contained 8 \mu l of lysate, mRNA as indicated in the figure legends, 320 nM C-terminal SECIS binding protein 2 and \textsuperscript{75}Se-labeled Sec-tRNA\textsuperscript{[Ser]Sec} or inorganic \textsuperscript{75}Se as indicated. Radiolabeled proteins were resolved by 12% SDS-PAGE followed by Phosphorimaging.

\textbf{Disclosure of potential conflicts of interest}

No potential conflicts of interest were disclosed.

\textbf{Funding}

This work was supported by a grant from the National Institutes of Health, GM077073 to P.R.C. and in part by the Intramural Research Program of the National Institutes of Health, NCI, Center for Cancer Research to D.L.H.

\textbf{ORCID}

Paul R. Copeland \textsuperscript{16} http://orcid.org/0000-0003-3343-0587

\textbf{References}

[1] Carlson BA, Lee BJ, Tsuji PA, Tobe R, Park JM, Schweizer U, Gladyshev VN, & Hatfield DL. Selenocysteine tRNA [Ser] Sec: From Nonsense Suppressor tRNA to the Quintessential Constituent in Selenoprotein Biosynthesis. In: Hatfield DL, Schweizer U, Tsuji PA, Gladyshev VN, editors. Selenium: Its molecular biology and role in human health, New York, NY: Springer; 2016, p. 3-12

[2] Kim I.K, Matsufuji T, Matsufuji S, Carlson BA, Kim SS, Hatfield DL, Lee BJ. Methylation of the ribosyl moiety at position 34 of selenocysteine (tRNA[Ser]Sec) is governed by both primary and tertiary structure. RNA 2000; 6 (9):1306-15; PMID:10999607; https://doi.org/10.1017/S1355838200000388

[3] Diamond AM, Choi IS, Crain PF, Hashizume T, Pomerantz SC, Cruz R, Steer CJ, Hill KE, Burk RF, McCluskey JA., et al. Dietary selenium affects methylation of the wobble nucleoside in the anticodon of selenocysteine tRNA([Ser]Sec). J Biol Chem 1993; 268 (19):14215-23; PMID:8314785

[4] Sturchler C, Westhof E, Carbon P, Krol A. Unique secondary and tertiary structural features of the eucaryotic selenocysteine tRNA(Sec). Nucleic Acids Res 1993; 21 (5):1073-9; PMID:8464694; https://doi.org/10.1093/nar/21.5.1073
[5] Klassen R, Ciftci A, Funk J, Bruch A, Butter F, Schaffrath R. tRNA anticodon loop modifications ensure protein homeostasis and cell morphogenesis in yeast. Nucleic Acids Res 2016; 44(22):10946-59

[6] Rezgui VAN, Tyagi K, Ranjan N, Konevega AL, Mittenstaedt J, Rodnina MV, Peter M, Pedrioli PG. tRNA (kUUU, tQUUG, and tEUUC) wobble position modifications fine-tune protein translation by promoting ribosome A-site binding. Proc Natl Acad Sci 2013; 110(30):12289-94; https://doi.org/10.1073/pnas.1300781110

[7] Tobe R, Naranjo-Suarez S, Everley RA, Carlson BA, Turanov AA, Tsuji PA, Yoo MH, Gygi SP, Gladyshev VN, Hatfield DL. High error rates in selenocysteine insertion in mammalian cells treated with the antibiotic doxycycline, chloramphenicol, or geneticin. J Biol Chem 2013; 288(21):14709-15; PMID:23589299; https://doi.org/10.1074/jbc.M112.446666

[8] Warner GJ, Berry MJ, Moustafa ME, Carlson BA, Hatfield DL, Faust JR. Inhibition of selenoprotein synthesis by selenocysteine tRNA[Ser]Sec lacking isopen-tenylenadenosine. J Biol Chem 2000; 275(36):28110-9; PMID:10821829

[9] Moustafa ME, Carlson BA, El-Saadani MA, Kryukov GV, Sun QA, Harney JW, Hill KE, Combs GF, Feigenbaum L, Mansur DB, et al. Selective inhibition of selenocysteine tRNA maturation and selenoprotein synthesis in transgenic mice expressing isopen-tenylenadenosine-deficient selenocysteine tRNA. Mol Cell Biol 2001; 21(11):3840-52; PMID:11340175; https://doi.org/10.1128/MCB.21.11.3840-3852.2001

[10] Carlson BA, Xu XM, Gladyshev VN, Hatfield DL. Selective rescue of selenoprotein expression in mice lacking a highly specialized methyl group in selenocysteine tRNA. J Biol Chem 2005; 280(7):5542-8; PMID:15611090; https://doi.org/10.1074/jbc.M411725200

[11] Kim JY, Carlson BA, Xu XM, Zeng Y, Chen S, Gladyshev VN, Lee BJ, Hatfield DL. Inhibition of selenocysteine tRNA(ASer)Sec aminoacylation provides evidence that aminoacylation is required for regulatory methylation of this tRNA. Biochem Biophys Res Commun 2011; 409 (4):814-19; PMID:21624347; https://doi.org/10.1016/j.bbrc.2011.05.096

[12] Songe-Møller L, van den Born E, Leihne V, Vágbo CB, Kristoffersen T, Krokan HE, Kirpekar F, Falnes PO, Klungland A. Mammalian ALKBH8 possesses tRNA methyltransferase activity required for the biogenesis of multiple wobble uridine modifications implicated in translational decoding. Mol Cell Biol 2010; 30(7):1814-27; PMID:20123966; https://doi.org/10.1128/MCB.01602-09

[13] Fradejas N, Carlson BA, Rijntjes E, Becker NP, Tobe R, Schweizer U. Mammalian Trtl1 is a tRNA[ASer]Sec-isopentenyl-transferase required for full selenoprotein expression. Biochim J 2013; 450(2):427-32; PMID:23289710; https://doi.org/10.1042/BJ20121713

[14] Carlson BA, Moustafa ME, Sengupta A, Schweizer U, Shrimali R, Rao M, Zhong N, Wang S, Feigenbaum L, Lee BJ, et al. Selective restoration of the selenoprotein population in a mouse hepatocyte selenoproteinless background with different mutant selenocysteine tRNAs lacking Um34. J Biol Chem 2007; 282 (45):32591-602; PMID:17848557; https://doi.org/10.1074/jbc.M707036200

[15] Sunde RA. Selenoproteins: Hierarchy, Requirements, and Biomarkers. In: Hatfield DL, Gladyshev VN, Berry MJ, editors. Selenium: Its molecular biology and role in human health, New York, NY: Springer; 2012, p. 137-52

[16] Moriarty PM, Reddy CC, Maquat LE. Selenium deficiency reduces the abundance of mRNA for Se-dependent glutathione peroxidase 1 by a UGA-dependent mechanism likely to be nonsense codon-mediated decay of cytoplasmic mRNA. Mol Cell Biol 1998; 18 (5):2932-9; PMID:9566912; https://doi.org/10.1128/MCB.18.5.2932

[17] Weiss SL, Sunde RA. cis-acting elements are required for selenium regulation of glutathione peroxidase-1 mRNA levels. RNA 1998; 4(7):816-27; PMID:9671054; https://doi.org/10.1017/S1355838298971990

[18] Sun X, Moriarty PM, Maquat LE. Nonsense-mediated decay of glutathione peroxidase 1 mRNA in the cytoplasm depends on intron position. EMBO J 2000; 19 (17):4734-44; PMID:10970865; https://doi.org/10.1093/emboj/19.17.4734

[19] Hill KE, Lyons PR, Burk RF. Differential regulation of rat liver selenoprotein mRNAs in selenium deficiency. Biochem Biophys Res Commun 1992; 185(1):260-3; PMID:1599462; https://doi.org/10.1016/S0006-291X(05)80984-2

[20] Lei XG, Evenson JK, Thompson KM, Sunde RA. Glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase are differentially regulated in rats by dietary selenium. J Nutr 1995; 125(6):1438-46; PMID:7782896

[21] Sunde RA, Raines AM, Barnes KM, Evenson JK. Selenium status highly regulates selenoprotein mRNA levels for only a subset of the selenoproteins in the selenoproteome. Biosci Rep 2009; 29(5):329-38; PMID:19076066; https://doi.org/10.1042/BSR20080146

[22] Howard MT, Carlson BA, Anderson CB, Hatfield DL. Translational redefinition of UGA codons is regulated by selenium availability. J Biol Chem 2013; 288(27):19401-13; PMID:23696641; https://doi.org/10.1074/jbc.M112.481051

[23] Chittum HS, Hill KE, Carlson BA, Lee BJ, Burk RF, Hatfield DL. Replenishment of selenium deficient rats with selenium results in redistribution of the selenocysteine tRNA population in a tissue specific manner. Biochim Biophys Acta 1997; 1359(1):25-34; PMID:9398082; https://doi.org/10.1016/S0006-291X(97)00092-X

[24] Hatfield D, Lee BJ, Hampton L, Diamond AM. Selenium induces changes in the selenocysteine tRNA[Ser]Sec
population in mammalian cells. Nucleic Acids Res 1991; 19(4):939-43; PMID:2017375; https://doi.org/10.1093/nar/19.4.939

[25] Kelmers AD, Heatherly DE. Columns for rapid chromatographic separation of small amounts of tracer-labeled transfer ribonucleic acids. Anal Biochem 1971; 44 (2):486-95; PMID:4943341; https://doi.org/10.1016/0003-2697(71)90236-3

[26] Hatfield D, Matthews CR, Rice M. Aminoacyl-transfer RNA populations in mammalian cells chromatographic profiles and patterns of codon recognition. Biochim Biophys Acta 1979; 564(3):414-23; PMID:259017; https://doi.org/10.1016/0005-2787(79)90032-7

[27] Carlson BA, Hatfield DL. Transfer RNAs that insert selenocysteine. Methods Enzymol 2002; 347:24-39

[28] Carlson BA, Novoselov SV, Kumaraswamy E, Lee BJ, Anver MR, Gladyshev VN, Hatfield DL. Specific excision of the selenocysteine tRNA[Ser]Sec (Trsp) gene in mouse liver demonstrates an essential role of selenoproteins in liver function. J Biol Chem 2004; 279 (9):8011-7; PMID:14660662; https://doi.org/10.1074/jbc.M310470200

[29] Mehta A, Rebsch CM, Kinzy SA, Fletcher JE, Copeland PR. Efficiency of mammalian selenocysteine incorporation. J Biol Chem 2004; 279(36):37852-9; PMID:15229221; https://doi.org/10.1074/jbc.M404639200

[30] Gupta N, Demong LW, Banda S, Copeland PR. Reconstitution of selenocysteine incorporation reveals intrinsic regulation by SECIS elements. J Mol Biol 2013; 425 (14):2415-22; PMID:23624110; https://doi.org/10.1016/j.jmb.2013.04.016

[31] Shetty SP, Shah R, Copeland PR. Regulation of selenocysteine incorporation into the selenium transport protein, Selenoprotein P. J Biol Chem 2014; 289 (36):25317-26; PMID:25063811; https://doi.org/10.1074/jbc.M114.590430

[32] Sawasaki T, Ogasawara T, Morishita R, Endo Y. A cell-free protein synthesis system for high-throughput proteomics. Proc Natl Acad Sci U S A 2002; 99 (23):14652-7; PMID:12409616; https://doi.org/10.1073/pnas.232580399

[33] Hadley KB, Sunde RA. Selenium regulation of thioredoxin reductase activity and mRNA levels in rat liver. J Nutr Biochem 2001; 12(12):693-702; PMID:12031252; https://doi.org/10.1016/S0955-2863(01)00189-9

[34] Gladyshev VN, Factor VM, Housseau F, Hatfield DL. Contrasting patterns of regulation of the antioxidant selenoproteins, thioredoxin reductase, and glutathione peroxidase, in cancer cells. Biochem Biophys Res Commun 1998; 251(2):488-93; PMID:9792801; https://doi.org/10.1006/bbrc.1998.9495