Translational Redefinition of UGA Codons Is Regulated by Selenium Availability

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Background: Selenium is incorporated into selenoproteins as the amino acid, selenocysteine. Incorporation of selenium into ~25 mammalian selenoproteins occurs by translational recoding whereby in-frame UGA codons are redefined to encode the selenium containing amino acid, selenocysteine (Sec). Here we applied ribosome profiling to examine the effect of dietary selenium levels on the translational mechanisms controlling selenoprotein synthesis in mouse liver. Dietary selenium levels were shown to control gene-specific selenoprotein expression primarily at the translation level by differential regulation of UGA redefinition and Sec incorporation efficiency, although effects on translation initiation and mRNA abundance were also observed. Direct evidence is presented that increasing dietary selenium causes a vast increase in ribosome density downstream of selenocysteine-encoding UGA codons for a subset of selenoprotein mRNAs and that the selenium-dependent effects on Sec incorporation efficiency are mediated in part by the degree of Sec-tRNA[Sec] suppression. Furthermore, we find evidence for translation in the 5′-UTRs for a subset of selenoproteins and for ribosome pausing near the UGA-Sec codon in those mRNAs encoding the selenoproteins most affected by selenium availability. These data illustrate how dietary levels of the trace element selenium can alter the readout of the genetic code to affect the expression of an entire class of proteins.

Selenium is a naturally occurring trace element that is essential for human health in small amounts but toxic at high levels. Human diseases associated with extreme selenium deficiency have been identified in geographic regions that are very low in selenium soil content, e.g. Keshan disease, a regional cardiomyopathy, which is endemic in regions of China (1). In regions of high selenium soil content, selenium toxicity can manifest as a condition called selenosis that is characterized by gastrointestinal upset, hair loss, fatigue, mood changes, and nerve damage (2, 3). Although disease symptoms resulting from severe selenium deficiency or intoxication are rare, there is strong evidence that less-overt variations in selenium status have multifaceted health effects including the risk of cancer, diabetes, skeletal muscle, and cardiac disease as well as maintenance of proper immune function (4).

One well established role of selenium is its incorporation into ~25 mammalian selenoproteins as the amino acid, selenocysteine (Sec) (5, 6). This is accomplished by an expansion of the normal rules of decoding to allow UGA codons to encode Sec in addition to its normal role in terminating translation. Ribosome reprogramming and the process of UGA-Sec codon redefinition is orchestrated by cis-acting Sec insertion sequence (SECIS) elements that reside in the 3′-UTR of selenoprotein mRNAs (7–10), as well as trans-acting factors including the SECIS-binding protein, SBP2 (11), and the Sec-specific elongation factor, eEFSec (12, 13), which ultimately delivers Sec-tRNA[Sec] to the ribosome (14).

In the context of selenoproteins, Sec mediates reductive/oxidation reactions on a number of substrates important for cellular redox homeostasis, thyroid hormone metabolism, protein folding, and disulfide formation or isomerization (4). It is also known that selenium availability can have a profound effect on selenoprotein levels with synthesis of certain essential housekeeping selenoproteins, thioredoxin reductase 1 (Txnrd1) and glutathione peroxidase 4 (Gpx4), for example, being more resistant to selenium limitation than stress-related selenoproteins, such as glutathione peroxidase 1 (Gpx1) (14–16). The “hierarchy” of differential selenoprotein expression in response to selenium availability and the specific substrates

2 The abbreviations used are: Sec, selenocysteine; RPF, ribosome-protected fragment; UGA-Sec, UGA selenocysteine codon; SECIS, Sec insertion sequence element; NMD, nonsense-mediated decay; mcmm, 5-methoxy carbonylmethyluridine; mcm5U, 5-methoxycarbonylmethyl-2′-O-methyl uridine; RPKM, reads per kilobase per million mapped reads for ribosome footprints; RPKM, reads per kilobase per million mapped reads for total RNA; CDS, coding sequence; Txnrd1, thioredoxin reductase 1; Gpx4, glutathione peroxidase 4; TE, translational efficiency; nt, nucleotides.

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and biochemical pathways affected likely account for the diversity of health effects associated with dietary selenium intake.

Despite the identification of key components of selenocysteine synthesis and incorporation, the mechanism by which dietary selenium affects selenoprotein synthesis remains controversial. One commonly invoked mechanism involves the nonsense-mediated decay (NMD) pathway in which, under selenium limiting conditions, the UGA-Sec codon in nonessential selenoprotein messages is recognized as a premature termination codon causing RNA degradation. For example, the reduction of Gpx1 mRNA levels under selenium deficiency has been shown to be due to post-transcriptional mRNA turnover rather than reduced transcription (17) by induction of the NMD pathway (18, 19), whereas Gpx4 mRNA levels are unaffected and resistant to NMD. Other explanations implicate changes in Sec insertion efficiency during translation due to differences in SECIS elements and their ability to recruit the Sec incorporation machinery (20–22). Also, it is known that selenium levels directly correlate with the degree of Um34 methylation of Sec-tRNA<sup>[Sec]</sup> wherein the Sec-tRNA<sup>[Sec]</sup> population consists of two isoforms, the non-Um34-containing isoform, 5-methoxycarbonylmethyluridine (mcm<sup>5</sup>U), and the Um34-containing isoform, 5-methoxycarbonylmethyl-2′-O-methyluridine (mcm<sup>5</sup>Um) (23). Based on the observation that the mcm<sup>5</sup>Um isoform is required for efficient expression of the stress-related selenoproteins but not for the housekeeping selenoproteins, it has been proposed that differential utilization of the two Sec-tRNA<sup>[Sec]</sup> isoforms during translation of selenoprotein mRNAs could account for selenium regulation of selenoprotein synthesis (24–26). Together these studies suggest that biosynthesis of selenoproteins is a complex process that may involve multiple levels of control; however, the degree of which each process may contribute to selenium-dependent regulation is unknown.

To directly examine the mechanisms of selenium-dependent regulation of selenoprotein expression in vivo, we have applied ribosome profiling, RNA-Seq, and traditional biochemical approaches to examine selenoprotein synthesis in liver. Using these approaches, we have defined the changes in mRNA abundance and translational activity that occur in response to altered dietary selenium intake and expression of a mutant Sec-tRNA<sup>[Sec]</sup> that prevents Um34 synthesis. Quantitative mapping of ribosome distribution at nucleotide resolution on selenoprotein mRNAs revealed that translational control of selenoprotein synthesis occurred primarily due to changes in Sec incorporation efficiency. Furthermore, translation of Gpx1, Sepx1, Sepw1, and Selh mRNAs requires the Sec-tRNA<sup>[Sec]</sup> mcm<sup>5</sup>Um isoform for efficient UGA-Sec decoding and involves an excess of ribosome protection upstream or at the UGA-Sec codon. Finally, we find an excess of ribosome-protected fragments upstream of the initiation codons for Selh, Sephs2, Txsrd1, and Selt. These studies provide a unique view of selenoprotein translational control and UGA redefinition efficiency in mammalian tissue and its regulation by dietary selenium intake.

**EXPERIMENTAL PROCEDURES**

Lyse Preparation—Three-week-old male WT and Trp<sup>A37G</sup> mice in a FVB/N background were fed diets supplemented with 0, 0.1, and 2.0 ppm selenium diets for 6 weeks before euthanasia. All animal work was approved under National Institutes of Health Institutional Animal Care and Use Committees protocol BRL-002. Livers were rapidly excised, cut into four equal parts, and immediately frozen in liquid nitrogen. For each experiment, 100 mg of frozen tissue was placed in a 2-ml tube containing a 1/8-inch-diameter stainless steel ball with 1.5 ml of pre-chilled lysis buffer (10 mM Tris-Cl (pH 7.5), 300 mM KCl, 10 mM MgCl<sub>2</sub>, 200 μg/ml cycloheximide (Sigma), 1 mM DTT, and 1% Triton X-100). Samples were homogenized by agitation at full speed for 45 s in the Mini-Beadbeater-8 (Biospec Products, Inc.).

Ribonuclease Digestion and Total RNA Preparation—To minimize biological variation, total RNA for RNA-Seq analysis and ribosome-protected fragments were prepared from the same lysate. 300 μl of crude lysate were immediately transferred to 2 ml of TRIzol reagent (Invitrogen), and total RNA was extracted according to the manufacturer’s specification. The remaining lysate was incubated with 500 units of RNase I (Invitrogen) for 30 min at 25 °C. Monosomes were isolated by centrifugation at 48,000 rpm for 2 h in a TL100 ultracentrifuge (Beckman Coulter). Ribonuclease-resistant RNA fragments were isolated from the pellet by TRIzol extraction and electrophoresed on a 15% polyacrylamide, 8M urea gel. The region of the gel containing 26–36-nucleotide-size RNA fragments was excised, and RNA was isolated by passive elution before library construction.

Ribosome Profiling and Library Construction—Gel-purified ribosome footprints and hydrolyzed total RNA fragments were treated with 5 units of Antarctic phosphatase (New England Biolabs) in the presence of 40 units of RNase inhibitor (Ambion) for 30 min at 37 °C followed by 5 min at 65 °C to deactivate the enzyme. Fragments were then treated with 20 units of polynucleotide kinase (New England Biolabs) for 60 min at 37 °C and purified using RNEasy MinElute columns (Qiagen) according to the manufacturer’s recommendations for small RNA. RPF and total RNA libraries were built using the TruSeq Small RNA Sample preparation kit (Illumina) according to the manufacturer’s specifications with rRNA subtraction using Ribo-Zero (Epicyter). Limited PCR was used to enrich for ligated fragments.

Illumina Sequencing and Processing of Reads—Library aliquots were run on an Agilent 2100 DNA high sensitivity bioanalyzer chip to validate the library size range. RPF and total RNA libraries were subjected to 50 cycles of single-end sequencing on an Illumina HiSeq 2000 instrument. Adapter sequences were removed using the Hannon laboratory FastX toolkit. rRNA sequences were removed using bowtie (27) alignment against mouse rRNA sequences, and all unaligned sequences were retained for further processing.

Alignment to RefSeq mRNA Sequences—RefSeq fasta sequences were obtained from the UCSC genome browser (mm9 assembly, RefSeq Genes were downloaded in November of 2012). These fasta files were reduced to a single entry for each
mRNA corresponding to the longest transcript isoform. For CDS alignments, the first 15 and last 3 codons were excluded to avoid bias caused by altered footprint density at the initiation and termination codons. Sequences that aligned to only a single site within the database, allowing for two mismatches, were identified using the bowtie short sequence aligner (27). Technical variability was addressed by linear regression analysis of all RefSeq mRNAs with greater than 0.01 RPKF or RPKM values (11,918 mRNAs) for WT samples obtained from mice fed diets supplemented with 0.1 or 2.0 ppm selenium. In each comparison R² values were >0.98, and >92% of mRNAs revealed less than a 2-fold difference in RPFKM or RPKM values (data not shown).

Ribosome profiling and total RNA sequences were aligned separately to selenoprotein and Gapdh mRNAs and were determined using the bowtie alignment parameters described above against the following RefSeq entries: NM_027652.2 Seli, NM_027905 Selo, NM_007860.3 Dio1, NM_013759.2 Sepx1, NM_024439.3 Sels, NM_015762.2 Txnrd1, NM_008160.6 Gpx1, NM_008162.2 Gpx4, NM_009155.3 Seppl1, NM_053102.2 Sep15, NM_013711.3 Txnrd2, NM_019979.2 Selk, NM_00104039.2 Selt, NM_009156.2 Sepw1, NM_009266.3 Sephs2, NM_030677.2 Gpx2, NM_001178058.1 Txnrd3, NM_029100.2 Sep1, NM_053267.2 Selm, NM_008161.3 Gpx3, NM_001033166.2 Selh, NM_172119.2 Dio3, NM_010050.2 Dio2, NM_175033.3 Selv, NM_008084.2 Gapdh.

Quantitative Analysis and A-site Assignment—The 5’ end of ribosome profiling and RNA reads were offset to the predicted first nucleotide of the A-site using the following rule: for sequence lengths ≤34 nt (+17 nt from the 5’ end of the sequence) and for sequence lengths ≥35 nt (+18 nt from the 5’ end of the sequence). These values were determined as the optimum position initiating ribosome P-sites at the AUG codon for all RefSeq mRNAs (see supplemental Figs. S1 and S3). Total mapped reads used to derive RPKM calculation were determined by aligning sequences to the CDSs of the RefSeq database described above. Total number of aligned reads were: WT 0 ppm selenium 23,945,053 (ribosome profiling) and 16,709,701 (RNA); WT 0.1 ppm selenium 30,296,837 (ribosome profiling) and 14,220,047 (RNA); WT 2.0 ppm selenium 16,709,701 (RNA); WT 0 ppm selenium 30,296,837 (ribosome profiling) and 14,220,047 (RNA); WT 0.1 ppm selenium 29,816,461 (ribosome profiling) and 14,220,047 (RNA); WT 2.0 ppm selenium 16,709,701 (RNA); WT 0 ppm selenium 23,945,053 (ribosome profiling) and 16,709,701 (RNA); WT 0.1 ppm selenium 30,296,837 (ribosome profiling) and 14,220,047 (RNA); WT 2.0 ppm selenium 16,709,701 (RNA); WT 0 ppm selenium 23,945,053 (ribosome profiling) and 16,709,701 (RNA); WT 0.1 ppm selenium 30,296,837 (ribosome profiling) and 14,220,047 (RNA); WT 2.0 ppm selenium 16,709,701 (RNA).

Modification Affect Global Translation of the Selenoproteome—Ribosome profiling involves the isolation and deep sequencing of ribosome-protected fragments (ribosome footprints or RPFs) after digestion of unprotected mRNA with RNase1 (29–31). Ribosome density and position on thousands of mRNAs can be determined by quantification and localization of ribosome footprints. An alternative method involving sucrose gradient fractionation of intact polysomes and microarray hybridization to determine mRNA position in the gradient was developed previously and applied to estimate overall ribosome density on mRNAs (32, 33). However, we selected ribosome profiling for analyzing selenoprotein expression due to its ability to measure both mRNA ribosome density and ribosome position at codon resolution. As previous in vitro studies have shown that UGA redefinition to encode Sec is in competition with termination (34, 35), we postulated that the ability to quantify ribosomes located 5’ and 3’ of UGA-Sec codons would provide a means of estimating changes in Sec incorporation efficiency and a more accurate method for measuring changes in full-length selenoprotein synthesis. Herein, we have applied this technique to examine the effects of altered dietary selenium levels and Sec-tRNA[Ser]Sec modification status on synthesis of the liver selenoproteome as outlined in Fig. 1A.

Um34 synthesis is the last step in maturation of tRNA[Ser]Sec and is dependent on an intact tertiary structure (36), prior synthesis of all base modifications, including isopentenyladenosine at position 37 (24, 26), and aminoacylation of the tRNA (37). When mammalian cells or tissues are selenium-deficient, the mcm²U isomser is enriched relative to the mcm²Um isomser, whereas the opposite is true under selenium sufficiency (Fig. 1B). A37G prevents Um34 synthesis (24, 26). The TrspA³⁻⁷G
transgenic mouse, which encodes 20 copies of the A37G transgene generates a Sec-tRNA^[Ser]Sec population consisting of a much higher proportion of mutant than wild type tRNA^[Ser]Sec (24).

We fed WT and Trsp^[A37G] mice defined diets supplemented with 0, 0.1, or 2.0 ppm selenium. Livers were harvested, and ribosome profiling experiments were performed as described under “Experimental Procedures.” Undigested total RNA was processed for RNA-Seq in parallel to estimate changes in mRNA abundance. Ribosome-protected fragments and total RNA fragments were aligned to an mRNA database derived from RefSeq. Analysis of tissue-derived ribosome footprint mRNA alignments to all CDSs longer than 1000 nt (supplemental Fig. S1A) revealed, as expected, that ribosome footprints were highly enriched in CDSs relative to the UTRs, and strong triplet phasing was observed corresponding to the step size of the ribosome. Importantly, no global bias was observed in the average ribosome protection toward the 3’ end of CDSs, indicating ribosomes were rapidly captured during tissue lysis. As has been previously reported (29, 31), a number of RPFs mapped to 5’-UTRs, likely reflecting ribosome occupancy of upstream open reading frames, and there was an elevation in RPFs found near the initiation codon. Thus, we show here that ribosome profiling in intact mammalian tissue captures the expected features of actively translating ribosomes.

The effects of altered dietary selenium and Sec-tRNA^[Ser]Sec modification on translation and mRNA abundance of the selenoproteome were examined by aligning ribosome footprint or total RNA sequence reads to the mRNA reference sequences for all 24 selenoproteins in mice. Ribosome density and mRNA...
abundance were assessed by counting the number of reads mapping to each mRNA corrected for the mRNA length and normalized to the total mapped reads for each sample (reads per kilobase per million mapped reads: RPKM for ribosome footprints and RPKM for total RNA fragments) (38). The global effect of dietary selenium levels on ribosome activity and mRNA abundance across the selenoproteome is shown as the cumulative ribosome footprint RPKM (Fig. 1C) and mRNA RPKM (Fig. 1D) for all selenoproteins with greater than 25 mapped ribosome-protected fragments or mRNA sequences in the sample derived from WT mice fed diets supplemented with 0.1 ppm selenium. The addition of 0.1 or 2.0 ppm selenium to the diets of WT mice increased global selenoprotein translation and mRNA abundance compared with the levels seen in mice fed selenium-deficient diets. Selenoprotein translational activity, mRNA abundance, and magnitude of response to increased dietary selenium were reduced in TrspA37G mice. Although both mRNA abundance and translational activity of the selenoproteome were affected, the greatest changes in response to increased dietary selenium levels in WT mice (~2–3×) was observed for ribosome footprint RPKFs, demonstrating that translational control plays a central role in the selenium-dependent regulation of the liver selenoproteome.

Selenium Availability and A37G Sec-tRNA\textsuperscript{Ser/Sec} Have Gene-specific Effects on Selenoprotein mRNA Abundance and Ribosome Density—We applied RNA-Seq to quantify selenoprotein mRNA abundance as a function of dietary selenium and Um34 methylation. The RNA RPKM measurements were first validated by comparison to quantitative real-time PCR (see supplemental Fig. S2). RPKM and quantitative real-time PCR measurements for the selenoprotein mRNAs in each sample were highly correlated ($r > 0.95$). Selenoprotein mRNA levels (Fig. 2A) span nearly 3 orders of magnitude with selenoprotein P (Sepp1), Gpx1, selenophosphate synthetase 2 (Sephs2), and Sepx1 being highly abundant in mouse liver. In WT mice, Gpx1, Sepw1, and selenoprotein H (Selh) mRNA levels were most sensitive to dietary selenium availability with the addition of 0.1 or 2.0 ppm selenium to diets, resulting in a >2-fold increase in mRNA abundance (Fig. 2C and supplemental Fig. S2). In selenium-deficient TrspA37G mice, the abundance of these three mRNAs was reduced relative to that seen in selenium-deficient WT mice, and RNA levels were restored to varying degrees by the addition of selenium to the diet. Dio1 showed a marked decrease in mRNA abundance in selenium-deficient TrspA37G mice that was increased by ~5-fold in the TrspA37G mutants fed selenium-supplemented diets. An effect of this magnitude on Dio1 mRNA was not observed in samples from WT mice.

Next we examined translational activity on selenoprotein mRNAs. As Sec incorporation efficiency is not 100%, we surmised that ribosome density downstream of the UGA-Sec codon would provide the most accurate measure of translational activity leading to the production of full-length protein. CDS RPKF measurements downstream of the UGA-Sec codon are shown in Fig. 2B and supplemental Fig. S2. For some selenoprotein mRNAs, the UGA-Sec codon is near the 3′ end of the CDS (selenoprotein S (SelS), selenoprotein O (SelO), Txnrd1, Txnrd2, Txnrd3, and selenoprotein K (SelK)). In these cases, ribosome footprint RPKFs were calculated for the entire CDS and, consequently, may not reflect the production of functional full-length Sec-containing protein due to the fraction of ribosomes that may terminate at the UGA-Sec codon. For all selenoproteins with non-C-terminal UGA-Sec codons the exception of SelK (discussed below) and Gpx3, there was an increase in RPKFs downstream of the UGA-Sec codon in WT mice that ranged from ~2 to nearly 30-fold in mice fed selenium-supplemented diets relative to those on selenium-deficient diets (Fig. 2D). The largest increases were seen for Gpx1 (13× and 16×), Sepx1 (6× and 8×), Sepw1 (7× and 11×), and Selh (21× and 27×). Similarly, selenium addition to the diets of TrspA37G mice resulted in an increase in 3′ ribosome footprint RPKFs, although in many cases to a lesser extent than seen in WT mice. Notably, there was a substantial reduction in the total RPKFs in TrspA37G mice fed selenium-supplemented diets relative to their WT counterparts for Gpx1, Sepx1, Sepw1, Selh (and to a lesser extent for Sepp1, Sephs2, Selenoprotein T (SelT), and Gpx3), suggesting that Um34 methylation was required to varying degrees for efficient synthesis of these selenoproteins. In contrast, 3′ RPKFs in TrspA37G mice were restored to WT levels by selenium supplementation for Gpx4, Sep15, and Dio1.

The relative selenium-dependent change in abundance of the three proteins in which 3′ RPKFs were significantly reduced in the TrspA37G mutant (Gpx1, Sepw1, Sepx1) and four where RPKFs were unaffected or affected to a lesser degree (Gpx4, Sep15, SelT, Txnrd1) was determined by Western blot analysis (Fig. 3). Comparisons to the changes in RPKFs confirmed qualitatively similar results supporting the validity of utilizing ribosome profiling to approximate changes in selenoprotein levels.

Sec Incorporation Efficiency Accounts for the Selenium-dependent Translational Control of Selenoprotein Synthesis—Ribosome footprint RPKFs reflect the number of ribosomes actively synthesizing protein for each mRNA, whereas translational efficiency (TE) (the amount of ribosome footprints normalized to mRNA abundance, RPKF/RPKM) provides a means of estimating translational regulation. In Fig. 4A, the TE across the CDS is shown for abundant selenoproteins in liver. A broad range of TEs is observed with selenoproteins with apparent high translational efficiency (see below), whereas Sepp1, Sephs2, and Sepw1 mRNAs are mRNAs that appear to have low translational efficiency. Assuming that the average translation elongation rate preceding and following the UGA-Sec codon is constant, CDS TE is primarily determined by both the efficiency of translation initiation and the efficiency of Sec insertion. As Sec incorporation efficiency is not 100% and it is conceivable that slow decoding of the UGA-Sec codon could slow the elongation rates of upstream ribosomes, the overall CDS TE may be strongly influenced by both the efficiency of Sec incorporation and the position of the UGA-Sec codon within the CDS (Fig. 4B), thus making interpretation of whole CDS RPKF values challenging. To circumvent these limitations, we determined the TE for regions upstream (5′ TE) and downstream (3′ TE) of the UGA-Sec codon, independently. However, for some selenoproteins it was not possible to obtain TE measurements either because the UGA-Sec codon was too near the 5′ or 3′ ends or the interro-
The gated region had RPKM or RPFKM values below a threshold value of 10 ("Experimental Procedures" and Ref. 26).

The selenoproteins revealed a range of TEs, with Selt and Gpx4 having the highest TE across all dietary selenium levels in both WT and Trsp<sup>A37G</sup> mice, whereas Gpx1 and Selk are examples of selenoproteins with low 5′ TEs (Fig. 5A). The addition of 0.1 or 2.0 ppm selenium resulted in less than a 2-fold increase in 5′ TE relative to selenium-deficient WT mice, except for Gpx1 in WT mice fed 2.0 ppm selenium diets, where the 5′ TE increased 2.6-fold, and for Selk in Trsp<sup>A37G</sup> mice fed 0.1 and 2.0 ppm selenium diets, where the 5′ TE increased 2.3- and 2.4-fold, respectively (Fig. 5C). Furthermore, the 5′ TE of Sepx1 in Trsp<sup>A37G</sup> mice was reduced by 0.63-, 0.33-, and 0.37-fold compared with selenium-deficient WT mice, suggesting that the mcm5U isoform of Sec-tRNA<sup>[Ser]</sup> may have an effect on either translation initiation or elongation rates upstream of the UGA-Sec codon of Sepx1.

In contrast to the relatively small changes in 5′ TE, a complex pattern of selenium-dependent changes in 3′ TE was observed reflecting changes in Sec incorporation efficiency depending on...
both dietary selenium levels and Um34 methylation (Fig. 5, B and D). For all mRNAs examined with the exception of Seli in Trsp<sup>A37G</sup> mice, the 3′ TE was reduced compared with the 5′ TE, indicating that Sec incorporation is not 100% efficient (compare Fig. 5, A to B). In WT mice, the addition of selenium to the diet increased 3′ TEs for Gpx1, Sepx1, Sepw1, Sephs2, Dio1, Gpx4, Sep15, and Selt from ~2-fold to nearly 8-fold (Fig. 5D). In contrast, we found the opposite effect for Seli, where the 3′ TE for Seli was slightly decreased in WT mice fed selenium-supplemented diets and increased under all selenium dietary levels in Trsp<sup>A37G</sup> mice (3.1-fold in diets with 0 ppm selenium, 2.4-fold with 0.1 ppm selenium, and 1.3-fold with 2.0 ppm selenium, compared with WT selenium-deficient mice). In the Trsp<sup>A37G</sup> mouse samples, three selenoprotein mRNAs, Gpx4, Sep15, and Selt, showed a selenium-dependent increase in 3′ TE to approximately the same levels as was observed in WT mice. In contrast, Gpx1, Sepx1, Sepw1, Dio1, Sephs2, and Selt showed a reduced response to dietary selenium supplementation in the Trsp<sup>A37G</sup> mouse samples, suggesting that translation of these four mRNAs requires Um34 methylation (to varying degrees) for efficient Sec incorporation and translational regulation by dietary selenium.

**Ribosome Density Near the UGA-Sec Codon and in the 5′-UTR**—Slow decoding of UGA-Sec codons resulting in ribosome pausing has been postulated to be a kinetic feature of Sec incorporation (34, 39). Here we find little evidence of excess ribosome footprints near UGA-Sec codons with the notable exceptions of four selenoprotein mRNAs, Gpx1, Sepx1, Sepw1, and Selh, where the number of ribosome footprints either at the UGA codon or in the 5 preceding codons exceeded 20% of the total footprints mapping to the respective mRNA (Table 1). Fig. 6 shows the ribosome footprint coverage across the mRNA (Fig. 6, A, C, E, and G) and the A-site codon location (Fig. 6, B, D, F, and H) of ribosome footprints mapping near the UGA-Sec codon for these mRNAs. Only for Sepx1 were there a large number of footprints with A-sites mapping to the UGA-Sec codon (Fig. 6D). The majority of footprints mapped to the −2 codon relative to the UGA-Sec codon for Gpx1 (Fig. 6B) and to the −4 codon for Sepw1 and Selh (Fig. 6, F and H).

**FIGURE 3.** Comparison of Western blots and ribosome profiling measurements of selenoprotein synthesis. Western blots of Gpx1, Sepw1, Sepx1, Gpx4, Sep15, Selt, and Txnrd1 from two biological samples are shown above the corresponding RPFKM (ribosome footprint reads/kilobase/million mapped reads) values in shaded boxes. For each selenoprotein, RPFKM values are shaded according to a continuous scale from 0 RPFKM (white) to the highest RPFKM value for that gene (dark gray). RPFKM values are for the portion of the mRNA downstream of the UGA-Sec codon except for Txnrd1, where the RPFKM value is for the CDS (RPFKM*). CBB, Coomassie Blue staining.

**FIGURE 4.** Translational efficiency of selenoprotein coding sequences. A, translational efficiency was calculated as the ribosome footprint RPFKM/RNA RPKMs across each CDS for WT and Trsp<sup>A37G</sup> mice fed diets supplemented with 0, 0.1, or 2.0 ppm selenium diets. B, relative length and position of the UGA-Sec codon (vertical bars) are shown for each selenoprotein.
To confirm accurate assignment of A-site codons, ribosome footprints were mapped to the Xbp1 mRNA, which is known to have a translational pause site at Asn-256 (31, 40), and this site is found to be a predominant site of ribosome pausing in our experiment (supplemental Fig. S3). The A-site relative to AUG initiation codons and UGA termination codons of RefSeq mRNAs are also shown in supplemental Fig. S3. The A-site codons start abruptly at the /H11001 codon (the AUG is in the P-site during initiation) and stop at the codon immediately preceding UGA termination codons. Ribosome footprint size was also examined at UGA-Sec codons to determine if the size was altered in such a way that it might affect A-site assignment at UGA-Sec codons. The results show that altered footprint size was not skewed relative to the size of ribosome footprints at other CDS locations.

Finally, we observed elevated mRNA protection in the 5′-UTR of several selenoprotein mRNAs. The highest levels of 5′-UTR protection were observed for Selh, Sephs2, Txnrd1, and Selt, where the fraction approached or exceeded 10% that of the total ribosome-protected fragments mapping to each respective mRNA (Table 2). Ribosome footprint coverage of 5′-UTRs is shown for Selh (Fig. 6G) as well as Sephs2 and Selt (Fig. 7).

FIGURE 5. Translational efficiency 5′ and 3′ of UGA-Sec codons. Translational efficiency (RPFKM/RPKM) of selenoprotein-coding sequences located either 5′ (A) or 3′ (B) of UGA-Sec codon (5′ or 3′ of the first UGA-Sec codon for Sepp1) in samples from WT and Trsp (A37G) mice fed diets supplemented with 0, 0.1, or 2.0 ppm selenium. C, same as A, expressed as the log 2 change in 5′ TE relative to the corresponding values measured in WT mice fed selenium-deficient diets. D, same as B expressed as the log 2 change in 3′ TE relative to the corresponding values measured in WT mice fed selenium-deficient diets. Selenoprotein mRNAs were excluded from this analysis (n.d.) if 5′ or 3′ RPKM values were <10 in Wt-0.1 sample or if the UGA-Sec was located near the start (5′ TE) or stop codon (3′ TE).
Regulation of the selenoproteome by dietary selenium intake is a multifaceted process involving gene-specific changes in both mRNA abundance and ribosome activity (Fig. 1). We find that selenoprotein mRNA levels in WT mouse liver are relatively resistant to changing levels of dietary selenium with the notable exception of Gpx1, Sepw1, and Selh in both WT and Trsp<sub>A37G</sub> mice as well as Dio1 in selenium-deficient Trsp<sub>A37G</sub> mice (Fig. 2 and supplemental Fig. S2). Although changes in transcriptional regulation by dietary selenium cannot be excluded as a contributing factor, we suggest that altered rates of mRNA turnover and, more specifically, susceptibility to mRNA turnover, not transcription, when selenium levels are altered (42). Dio1 may be an exceptional case, as a reduction in mRNA abundance was only observed in selenium-deficient Trsp<sub>A37G</sub> mice. The three deiodinases in mammals (the other two deiodinases, Dio2 and Dio3, were below detection limits in liver) are involved in the biosynthesis and interconversion of thyroid hormones between the inactive and active form, T4 and T3, respectively (43). As the Dio1 gene contains thyroid hormone response promoter elements (44), it is a distinct possibility that altered levels of the active T3 hormone, due to changing deiodinase activity in liver or other tissues, may create a feedback loop affecting transcription of Dio1 that may in part contribute to the large decrease in Dio1 mRNA observed in selenium-deficient Trsp<sub>A37G</sub> mice.

We find that the selenoprotein mRNAs in liver that reveal the largest changes in abundance due to altered dietary selenium levels (Gpx1, Sepx1, Sepw1, and Selh) are the same as those that reveal the greatest changes in active translation and Sec incorporation efficiency. The data presented in Fig. 5 further clarify the mechanism of translational control. Although selenium availability appears to have modest effects on translation initiation estimated by changing translational efficiency upstream of the UGA-Sec codon, we find dynamic selenium-dependent changes in ribosome density downstream of the UGA-Sec codon, supporting a model in which Sec incorporation is a limiting step during biosynthesis of selenoproteins and a primary target for selenium regulation of selenoprotein expression.

It has been previously reported that the selenium-induced methylation of Um34 is required for expression of a subset of stress-related selenoproteins (see Fig. 3 and Refs. 24 and 25). Here we extend these findings by showing that either selenium deficiency or expression of an A37G mutant of Sec-tRNA<sub>[Ser]<sup>Sec</sup></sub> severely reduced ribosome density downstream of the UGA-Sec codon for Gpx1, Sepx1, Sepw1, and Selh, reflecting diminished Sec incorporation efficiency. In addition, under selenium-adequate conditions, Sec insertion efficiency remains relatively unaffected by expression of the A37G mutant Sec-tRNA<sub>[Ser]<sup>Sec</sup></sub> for the housekeeping selenoprotein mRNAs, including Gpx4 and Sep15, with many selenoproteins showing intermediate changes. Selh is notable among the selenoproteins in that selenium-adequate or supplemented diets do not appear to increase Sec insertion efficiency in WT mice, whereas in Trsp<sub>A37G</sub> mice Sec insertion efficiency is increased, suggesting the unexpected possibility that the unmethylated mcm5U Sec-tRNA<sub>[Ser]<sup>Sec</sup></sub> isoform may be preferentially utilized for UGA-Sec decoding of Selh, thus increasing its expression under selenium-limiting conditions. It should be noted that the UGA-Sec codon is near the termination codon of Selh, and thus ribosome density 3' of the UGA-Sec codon may also be influenced by the efficiency of termination. The Selh protein is a putative CDP-alcohol phosphatidyltransferase involved in the production of phospholipids (45), an activity that may be important for replacing damaged phospholipids due to oxidative stress induced by selenium deficiency.

Furthermore, we find evidence for a high level of ribosome protection immediately upstream of the UGA-Sec codons of Gpx1, Sepx1, Sepw1, and Selh, the same genes that are most affected by dietary selenium levels. Assignment of ribosome A-sites to the 3' end of RefSeq mRNAs (supplemental Fig. S3) demonstrates a sharp drop-off at the codon preceding standard UGA termination codons. Consequently, the accumulation of ribosomes immediately upstream of UGA-Sec codons must be interpreted with caution. In one scenario, ribosomes may pause at the UGA-Sec codon due to slow decoding, resulting in tightly stacked ribosomes (34, 46) behind the UGA-Sec codon. If ribosomes decoding UGA-Sec codons are not effectively captured by cycloheximide treatment and are released during tissue processing, this could allow for limited movement of the preceding paused ribosomes to positions immediately upstream of the UGA-Sec codon. Alternatively, ribosome pausing may occur before the UGA-Sec codon enters the A-site of the ribosome. In this case, slow decoding of upstream codons could allow time for assembly of the Sec incorporation machinery and reprogramming of ribosomes before decoding of the UGA-Sec codon, thus minimizing competition with termination. Several
FIGURE 6. **Ribosome footprints near UGA-Sec codons.** The number of ribosome footprints mapping to each nucleotide across the mRNAs of Gpx1 (A), Sepx1 (C), Sepw1 (E), and Selh (G) normalized to total mapped reads (Coverage) is shown for each mRNA in livers of WT or Trsp<sup>A37G</sup> (A37G) mice fed diets supplemented with 0, 0.1, or 2.0 ppm selenium. Below the histograms in A, C, E, and G is a schematic of each mRNA. Thin lines represent UTRs, boxes represent open reading frames, and red lines indicate the position of the UGA-Sec codons. The number of ribosome footprints with A-site codons assigned to the −5 to +3 codons relative to UGA-Sec, normalize to total mapped reads (reads per million mapped reads, rpm, is shown for Gpx1 (B), Sepx1 (D), Sepw1 (F), and Selh (H)).

TRANSLATIONAL CONTROL OF SEC INSERTION
previous studies have demonstrated that secondary structures downstream of UGA-Sec codons, which may slow local ribosome elongation rates, enhance Sec incorporation efficiency (47, 48). In addition, nascent peptides in the exit channel of the ribosome may provide an alternative mechanism to slow translation in the region preceding the UGA-Sec codon. Furthermore, additional caution is warranted due to library construction biases that may influence quantification of ribosome-protected fragments at any given position. Nevertheless, experimental analysis of the UGA-Sec sequence context of these mRNAs is warranted to illuminate the mechanisms contributing to increased ribosome protection and its contribution to selenium-dependent regulation of Sec incorporation during synthesis of these selenoproteins.

Increased ribosome protection was observed in the 5′-UTRs of several selenoproteins. For Selh, Sephs2, and Selt, a contiguous open reading frame exists between the 5′-UTR footprints and the annotated AUG codons, suggesting the possibility of N-terminal extensions to the selenoprotein or, alternatively, translation of peptides derived from small out-of-frame upstream upstream open reading frames. Extensive genome-wide translation of upstream open reading frames, initiated by both AUG and non-AUG codons, has recently been described in studies utilizing ribosome profiling in mammalian cells and in yeast (29–31). Although the fraction of total ribosome-protected fragments mapping to the 5′-UTR did have an inverse correlation with dietary selenium levels for Selh and Sephs2 in our experiments (Table 2), the relationship between 5′-UTR translation and selenium-dependent regulation of these selenoproteins remains unclear, and the effect is likely to be small based on our observation that 5′ TE, which should in part reflect initiation efficiency, is relatively unaffected by increasing selenium availability. Given the complex relationship between 5′-UTR translation and gene translation, it is possible that 5′-UTR translation is not related to dietary selenium levels but is utilized under other circumstances to regulate selenoprotein translation or to produce selenoproteins with N-terminal extensions.

The data presented here demonstrate that dietary selenium levels can alter the efficiency by which the standard rules of genetic decoding are redefined to allow UGA codons to encode Sec. Importantly, we show that UGA-Sec redefinition is inefficient and a primary determinant of selenoprotein synthesis rates in vivo. Our study provides direct support for a model in which the mcm5U and mcm5Um isoforms of Sec-tRNA[Sec] are differentially utilized to determine Sec incorporation efficiencies in a manner that depends upon the selenoprotein mRNA. Likely candidates for factors involved in discrimination between the two Sec-tRNA[Sec] isoforms include differences

**TABLE 2**

| Fraction of ribosome footprints in 5′-UTRs |
|------------------------------------------|
| The number of ribosome footprints with A-sites mapping within the 5′-UTR (excluding the two codons preceding the AUG codon) are shown as a percentage of the total ribosome footprints mapping to each respective selenoprotein gene. Data are shown for WT or TrspA37G (A37G) mice fed diets supplemented with 0, 0.1, or 2.0 ppm selenium. |
| Wt-0 | Wt-0.1 | Wt-2.0 | A37G-0 | A37G-0.1 | A37G-2.0 |
| Gpx1 0 0 0 0 0 0 |
| Selh 42 33 17 38 16 11 |
| Seph1 13 2 4 0 6 5 |
| Sepx1 5 4 5 4 4 5 |
| Dio1 0 0 0 0 0 0 |
| Gpx3 0 0 0 0 0 0 |
| Selt 9 8 13 8 8 10 |
| Sep15 4 2 1 4 2 1 |
| Selx 6 8 7 1 8 7 |
| Seppx1 7 4 5 4 4 4 |
| Gpx4 0 0 0 0 0 0 |
| Selx 1 1 2 1 1 1 |
| Sepox2 33 19 18 31 25 23 |
| Selo 0 0 0 0 0 0 |
| Sels 0 0 0 0 0 0 |
| Txnrd1 10 10 14 3 6 10 |
| Txnrd2 0 0 0 0 0 0 |
| Txnrd3 3 0 0 0 0 0 |

**FIGURE 7. Ribosome protection in the 5′-UTR.** The number of ribosome footprints mapping to each nucleotide across the 5′-UTR and coding sequence extending 10 nt beyond the UGA-Sec codon of Sephs2 (A) and Selt (B), normalized to total mapped reads (Coverage) is shown for each mRNA in livers of WT or TrspA37G (A37G) mice fed diets supplemented with 0, 0.1, or 2.0 ppm selenium. Below the histograms in A and B is a schematic of each mRNA. Thin lines represent the 5′-UTR, and boxed regions are the open reading frame up to, and extending 10 nt beyond the UGA-Sec codon. Red lines indicate the position of the UGA-Sec codon.
between SECIS elements, the local sequence context of the UGA-Sec codon, and their influence on either recruitment of Sec-tRNA[^Ser]Sec isofoms (23), the preferential utilization of one isoform over the other and the resulting effect of altered Sec insertion efficiency on mRNA abundance provides a unifying mechanism to explain how dietary selenium regulates the readout of the genetic code and gene-specific selenoprotein expression.

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