A regulatory role for Sec tRNA^{[Ser]Sec} in selenoprotein synthesis

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ABSTRACT

Selenium is biologically active through the functions of selenoproteins that contain the amino acid selenocysteine. This amino acid is translated in response to in-frame UGA codons in mRNAs that include a SECIS element in its 3' untranslated region, and this process requires a unique tRNA, referred to as tRNA^{[Ser]Sec}. The translation of UGA as selenocysteine, rather than its use as a termination signal, is a candidate restriction point for the regulation of selenoprotein synthesis by selenium. A specialized reporter construct was used that permits the evaluation of SECIS-directed UGA translation to examine mechanisms of the regulation of selenoprotein translation. Using SECIS elements from five different selenoprotein mRNAs, UGA translation was quantified in response to selenium supplementation and alterations in tRNA^{[Ser]Sec} levels and isoform distributions. Although each of the evaluated SECIS elements exhibited differences in their baseline activities, each was stimulated to a similar extent by increased selenium or tRNA^{[Ser]Sec} levels and was inhibited by diminished levels of the methylated isoform of tRNA^{[Ser]Sec} achieved using a dominant-negative acting mutant tRNA^{[Ser]Sec}. tRNA^{[Ser]Sec} was found to be limiting for UGA translation under conditions of high selenoprotein mRNA in both a transient reporter assay and in cells with elevated GPx-1 mRNA. This and data indicating increased amounts of the methylated isoform of tRNA^{[Ser]Sec} during selenoprotein translation indicate that it is this isoform that is translationally active and that selenium-induced tRNA methylation is a mechanism of regulation of the synthesis of selenoproteins.

Keywords: selenium; Sec; translation; tRNA; selenoprotein

INTRODUCTION

Selenium is an essential trace element and much of its influence on human health is likely mediated through its activity as a component of selenium-containing proteins. The genomes of higher mammals encode 25 distinct selenoproteins, some of which function in redox homeostasis, thyroid hormone metabolism, sperm structure, and selenium metabolism (Kryukov et al. 2003). Selenoproteins contain selenium in the form of the amino acid selenocysteine (Sec), which is incorporated during translation in response to UGA codons. This process requires several dedicated translation factors that serve to distinguish between UGA codons designated for Sec from those terminating translation.

The 3' untranslated region of all mammalian selenoprotein mRNAs contains a region of conserved secondary structure, the Sec insertion sequence (SECIS element). This

element is required for recognition of UGA codons as Sec, and serves as the binding site of the SECIS element binding protein-2 (SBP2; Copeland et al. 2000). SBP2 binds to a conserved, non-Watson-Crick base-paired region in the stem of the SECIS element (Fletcher et al. 2001) and remains bound through multiple cycles of selenoprotein translation (Low et al. 2000). SBP2 also binds to a selenoprotein-specific elongation factor, eEFSec (Tujebajeva et al. 2000) and may bind the tRNA (tRNA^{[Ser]Sec}) that serves as both the site of Sec synthesis from serine as well as the Sec adaptor molecule (Lee et al. 1989).

Individual selenoproteins are present in tissues at levels that may differ by more than an order of magnitude. The transcription of several selenoproteins responds to environmental cues, such as the presence of reactive oxygen species (Jornot and Junod 1997). Of particular interest is the regulation of selenoproteins by dietary intake of selenium. Under conditions of selenium deficiency, the mRNA levels for several selenoproteins are reduced as compared to selenium-adequate conditions (Bermano et al. 1995; Hadley and Sunde 2001) and this has been shown to occur by a process involving nonsense-codon-mediated decay of tran-

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scripts containing in-frame termination codons (Maquat 2001). However, this reduction in mRNA levels is less dramatic than the decline of selenoenzyme activities, indicating regulation of selenoprotein translation as well (Weiss et al. 1996; Weiss Sachdev and Sunde 2001). In addition, selenoproteins have been shown to react in a hierarchical manner with respect to their decline and replenishment over changing levels of selenium availability (Behne and Kyriakopoulos 1993). The mechanism of regulation of selenoprotein translation remains unclear.

One likely control point for the regulation of selenoprotein synthesis is the recognition of the appropriate UGA codon as Sec. UGA translation is relatively inefficient and is also responsive to selenium, as evidenced by studies showing a Sec-encoding UGA codon decreases polysome loading of selenoprotein mRNAs and is read through more efficiently following selenium supplementation (Fletcher et al. 2000; Martin and Berry 2001). Estimates of Sec translation efficiency range from 1% to 15% based on results of reporter assays using constructs containing either a UGA or UGU at the appropriate position (Kollmus et al. 1996). Although individual SECIS elements will differentially support UGA recognition as Sec, tRNA^{[Ser]Sec} may also have a regulatory role in this process.

tRNA^{[Ser]Sec} is aminoacylated with serine, which is subsequently converted to Sec, making it unique among tRNAs in that it serves as the site of synthesis of its cognate amino acid. At 90 nt in length, it is the longest characterized mammalian tRNA (Diamond et al. 1981), although it only contains four modified residues, including mcm⁵U at the wobble position of the anticodon (Diamond et al. 1993; Zhou et al. 1999). This modified residue may undergo 2'-O-methylation, yielding mcm⁵Um, a modified nucleotide not known to exist in any other tRNA (Diamond et al. 1993). The relative amounts of the unmethylated and methylated isoforms, referred to as mcm⁵U and mcm⁵Um, respectively, vary with different cell types and tissues (Hatfield et al. 1991; Chittum et al. 1997). In addition, selenium availability influences both the absolute and relative levels of the tRNA^{[Ser]Sec} isoforms, with selenium supplementation inducing a 25%–50% increase in the total tRNA^{[Ser]Sec} population as well as inducing a shift from mcm⁵U to mcm⁵Um (Hatfield et al. 1991; Chittum et al. 1997). In addition to the selenium-induced shift to the methylated isoform, additional data suggest that the distribution between these isoforms is highly regulated. Overexpression of tRNA^{[Ser]Sec} by more than fourfold results in the accumulation of mcm⁵U but not mcm⁵Um (Moustafa et al. 1998, 2001), and conversely, reduction in tRNA^{[Ser]Sec} gene copy number from 2 to 1 by homologous recombination reduces the population almost exclusive at the expense of mcm⁵U (Chittum et al. 1997). This maintenance of the levels of mcm⁵Um following these manipulations indicates possible distinct functions for each isoform.

In this article, the regulation of selenoprotein synthesis is

examined, with a focus on the recognition of the UGA as Sec. To accomplish this, a specialized, SECIS-driven reporter construct that permits the evaluation of UGA readthrough in experimentally defined cellular environments was used (Kollmus et al. 1996). The results support a regulatory role of tRNA^{[Ser]Sec} in selenoprotein translation as a function of both selenium availability and selenoprotein mRNA levels.

RESULTS

Chinese hamster ovary (CHO) cells were used as a cell culture model to examine the control of selenoprotein synthesis. These cells are relatively fast growing, are good recipient cells for transfection, and have already been characterized with regard to both the effects of selenium on selenoprotein induction as well as effects on tRNA^{[Ser]Sec}. The supplementation of the culture media of CHO cells with only 30 nM selenium, in the form of sodium selenite, results in the increase in the levels of selenoproteins, albeit to different degrees. For example, using biochemical assays generally accepted as being quantitative for the respective proteins, supplementation of the media of CHO cells with 30 nM Se results in a fourfold increase in GPx-1 ($p < 0.01$) but only a 54% increase in TR1 ($p < 0.05$; Fig. 1).

SECIS elements derived from different selenoprotein mRNAs support UGA translation with differing efficiencies

The translation of UGA codons as Sec is relatively inefficient, and is responsive to selenium supplementation (Fletcher et al. 2000). This process therefore represents a possible key regulatory step in selenoprotein synthesis. To examine UGA translation independent of other possible regulatory mechanisms, a specialized reporter construct was used. The pBPLUGA vector (Kollmus et al. 1996) contains coding regions for two reporter genes, β -galactosidase (β gal) and firefly luciferase (luc), separated by a linker containing an in-frame TGA codon. Translation termination at this UGA codon produces a peptide with only β gal activity. When a functional SECIS element is inserted into the polycloning site downstream from the luc coding region, translation of the UGA occurs, resulting in a fusion protein with both β gal and luc enzyme activities. Measurements of luc activity in cells transfected with pBPLUGA constructs represent the efficiency of UGA translation, whereas the determined β gal activity permits normalization for transfection efficiency. This reporter has been used by others to establish a hierarchy of SECIS function and to examine the effects of transiently altered levels of factors contributing to UGA translation (Kollmus et al. 1996; Wingler et al. 1999; Nasim et al. 2000).

SECIS elements from representative selenoprotein mRNAs were cloned into pBPLUGA, and luc and β gal activities were determined from extracts obtained from CHO cells

FIGURE 1. GPx-1 and TR levels are influenced by selenium and tRNA^{[Ser]Sec} levels. CHO cells overexpressing tRNA^{[Ser]Sec} (ST4) or a dominant-negative A37G tRNA^{[Ser]Sec} mutant whose expression results in reduced levels of mcm⁵Um (i6A⁻) were grown in standard medium or medium supplemented with 30 nM selenium for 3 d and the indicated selenoprotein levels were determined. Values are the average of three independent lysates $\pm$ standard deviation. (A) Glutathione peroxidase activity was measured by a coupled spectrophotometric assay and activity is expressed as nanomoles NADPH per minute per milligram of protein. (B) Thioredoxin reductase activity was measured from partially purified protein extracts by the reduction of sulphydryl groups in DNTB. Activity is expressed as micromoles of NTB reduced per micromole of protein.

transiently transfected with the generated constructs. Selected SECIS elements included those derived from the genes for the cytosolic glutathione peroxidase (GPx-1), thioredoxin reductase 1 (TR1), allelic variants of Sep15 (containing T and A, or C and G at positions 811 and 1125, respectively), and mitochondrial thioredoxin reductase (TR3).

CHO cells transiently transfected with the pBPLUGA construct lacking a SECIS element exhibit low levels of luc activity (Fig. 2), representing inefficient SECIS-independent UGA readthrough. This result is in agreement with a pre-

vious report indicating that UGA can be suppressed by both tRNA^[Trp] and serine-aminoacylated tRNA^{[Ser]Sec} (Jung et al. 1994). In contrast, transfection of CHO cells with constructs containing the above described SECIS elements resulted in substantial luc activity, 8- to 50-fold higher than that observed using the vector alone (Fig. 3). The SECIS element from GPx-1 displayed efficiency approximately threefold greater than the SECIS elements from TR1 and TR3. The Sep15 TA sequence displayed 1.9-fold greater efficiency than the form containing the Sep15 CG haplotype.

FIGURE 2. UGA suppression in CHO cells is affected by tRNA^{[Ser]Sec} levels, but not selenium or GPx-1 mRNA overexpression. CHO cells overexpressing wild-type tRNA^{[Ser]Sec} (ST4), A37G tRNA^{[Ser]Sec} mutant (i6A⁻), or elevated GPx-1 mRNA were transfected with the pBPLUGA reporter construct. Cells were transiently transfected and incubated for 3 d in standard medium or medium supplemented with 30 nM selenium. UGA suppression was measured as luciferase activity, achieved by the translation of the in-frame UGA codon separating the luc and β gal genes, and normalized by dividing by β gal activity. Values are the average of three independent lysates $\pm$ standard deviation.

Selenium stimulates UGA translation and methylation of tRNA^{[Ser]Sec}

To assess the effects of selenium supplementation on SECIS element function, the reporter constructs containing the five SECIS elements described above were transfected into CHO cells, and reporter activities were measured following supplementation of the medium with 30 nM selenium for 3 d. UGA translational efficiency achieved from all five constructs containing SECIS elements increased 2.2- to 3.4-fold following selenium supplementation (Fig. 4). This effect was SECIS dependent as translation in the absence of a SECIS element was not stimulated by selenium supplementation (Fig. 2). It is noteworthy that the relative induction achieved by selenium supplementation was similar for each of the different SECIS elements evaluated (Fig. 4).

Selenium has been reported to both increase the total levels of tRNA^{[Ser]Sec} and stimulate a redistribution of isoforms to mcm⁵Um in a variety of tissues and cell types, including CHO cells (Hatfield et al. 1991; Diamond et al. 1993; Mansur et al. 2000, 2001). The addition of 30 nM sodium selenite to the media of CHO cells results in a 50% increase in tRNA^{[Ser]Sec} levels, as determined by Northern blot analysis (data not shown), as well as a 78% increase in

FIGURE 3. SECIS elements from different selenoproteins support UGA translation with different efficiencies. CHO cells were transfected with the pBPLUGA reporter constructs containing SECIS sequences from five indicated selenoproteins. UGA translation was measured as luciferase activity achieved by the translation of the in-frame UGA codon separating the luc and β gal genes, and normalized by dividing by β gal activity. Values are the average of three independent lysates $\pm$ standard deviation.

the proportion of tRNA^{[Ser]^{Sec}} that is mcm⁵Um, from 22% to 39%, as determined by RPC-5 chromatography (Table 1). Because the same dose that causes these changes in the tRNA^{[Ser]^{Sec}} causes stimulation in UGA translation using the pBPLUGA reporter system, a causal relationship was investigated.

Levels and methylation of tRNA^{[Ser]^{Sec}} affect UGA translation

CHO cells were transfected with a DNA fragment containing the gene for tRNA^{[Ser]^{Sec}}, resulting in the generation of a transfectant that overexpresses tRNA^{[Ser]^{Sec}} by fourfold as determined by Northern blot analysis (data not shown). The relative distribution of tRNA^{[Ser]^{Sec}} isoforms in these cells, referred to as ST4, was determined by RPC-5 chromatography and shown to be similar to nontransfected CHO cells (Table 1). In contrast to what occurs when control CHO cells are incubated with selenium, selenium supplementation of the media of ST4 cells did not significantly change the isoform distribution (Table 1), consistent with previous data on selenium supplementation of tRNA^{[Ser]^{Sec}} overexpressing cells (Moustafa et al. 1998).

ST4 cells therefore afforded us the possibility of evaluating the consequences of tRNA^{[Ser]^{Sec}} levels on UGA translation independent of selenium status. Elevated tRNA^{[Ser]^{Sec}} levels resulted in increased UGA translation, with increases ranging from 1.6- to 2.9-fold above control cells for all five SECIS elements evaluated (Fig. 5). The stimulation observed did not increase appreciably when selenium was added to the culture media. In addition, SECIS-independent UGA suppression, assessed using the pBPLUGA vector, increased approximately fourfold in the presence of elevated tRNA^{[Ser]^{Sec}} (Fig. 2).

To investigate the role of mcm⁵Um in UGA translation, a dominant-negative acting mutant tRNA^{[Ser]^{Sec}} that does not form the modified nucleotide i⁶A at position 37 (due to the in vitro mutagenesis of that position for an A to a G) and does not undergo ribose methylation at mcm⁵U34 was used (Warner et al. 2000; Moustafa et al. 2001). A DNA fragment containing the gene of this derivative tRNA^{[Ser]^{Sec}} was introduced into CHO cells and a transfectant was selected for analysis. These cells, referred to as i⁶A⁻, expressed total tRNA^{[Ser]^{Sec}} (including endogenous and that derived from the transfected gene) at levels 25-fold above baseline (data not shown), and analysis by PCR and restriction endonuclease digestion confirmed the presence of the exogenous tRNA gene in the DNA of i⁶A⁻ cells (data not shown). The level of mcm⁵Um declined by 55% in i⁶A⁻ cells from that observed in control cells (Table 1). The i⁶A⁻ cells also exhibited an attenuated shift from mcm⁵U to mcm⁵Um following selenium supplementation, 20%, as compared to a 95% increment in control cells.

UGA translation in i⁶A⁻ cells was assessed using each of the reporter constructs and shown to be greatly diminished compared to control cells (Fig. 6). This effect was most pronounced for the Sep15(AT) and Sep15(GC) SECIS elements, which were reduced by approximately 95% of baseline activity. The activities of the TR1, TR3, and GPx-1 SECIS elements were reduced by 91%, 86%, and 80%, respectively, and reporter activity did not significantly increase with selenium supplementation, as was observed in control cells not containing the mutant tRNA (Fig. 6). UGA suppression was assessed using the pBPLUGA vector, and shown to decline by 65% in i⁶A⁻ cells compared to that observed in control cells ($p < 0.01$), and suppression was

FIGURE 4. Selenium stimulates UGA translational efficiency. CHO cells were transfected with the pBPLUGA reporter constructs containing SECIS sequences from five selenoproteins, followed by incubation for 3 d in standard medium or medium supplemented with 30 nM selenium. UGA translation was measured as luciferase activity achieved by the translation of the in-frame UGA codon separating the luc and β gal genes, and normalized by dividing by β gal activity. Values are the average percent increase over UGA translation in unsupplemented cells of 12 independent lysates $\pm$ standard deviation.

TABLE 1. Levels and distribution of tRNA^{[Ser]Sec} isoforms in transfected cells and following incubation in selenium-supplemented medium

Cells ^a	Isoform distribution ^b		
	mcm ⁵ U	mcm ⁵ Um	mcm ⁵ Um/mcm ⁵ U
Control	78	22	0.29
Control 1d Se	60	40	0.66
Control 3d Se	61	39	0.63
Control 5d Se	63	37	0.59
ST4	77	23	0.31
ST4 Se	76	24	0.32
i ⁶ A ⁻	90	10	0.12
i ⁶ A ⁻ Se	87	13	0.14
GPx7	49	51	1.03
GPx7 Se	29	71	2.41
GPx7 + tRNA	53	47	0.88
GPx7 + tRNA Se	46	54	1.18

^aControl cells stably transfected with the pLNCX vector, ST4 cells that overexpress tRNA^{[Ser]Sec}, i⁶A⁻ cells that express a dominantly acting derivative of tRNA^{[Ser]Sec}, and GPx7 cells that overexpress GPx-1 were incubated with 30 nM sodium selenite where indicated (Se). GPx7 + tRNA indicates that GPx7 cells were transfected with the tRNA^{[Ser]Sec} gene and these cells overexpressed the corresponding tRNA by 25-fold.

^bPercentage of mcm⁵U and mcm⁵Um within the tRNA^{[Ser]Sec} population.

also not stimulated following selenium supplementation (Fig. 2).

The contribution of UGA translation to the regulation of selenoprotein synthesis

By utilizing reporter constructs that permit the focus on the translation of UGA codons that direct the incorporation of Sec, the effects of the SECIS element, selenium availability, and tRNA^{[Ser]Sec} isoform levels and methylation were evaluated (see above). To assess the contribution of UGA translation to the overall control of selenoprotein synthesis, the effects of perturbations of the tRNA^{[Ser]Sec} population on the levels of selected selenoproteins were investigated. Two selenoproteins were selected for study, GPx-1 and TR1, chosen because the biochemical assays for these activities reflect protein levels and their levels in the i⁶A⁻ transgenic mouse have been described (Moustafa et al. 2001).

Levels of several selenoproteins have been shown to be reduced in i⁶A⁻ transgenic mice (Moustafa et al. 2001). Consistent with this observation, the levels of both GPx-1 and TR1 were significantly lower in CHO cells overexpressing the mutant tRNA, with GPx-1 activity reduced to 30% of the level in control cells (Fig. 1A, $p < 0.02$) and enzyme activity did not increase with selenium supplementation, in contrast to the large stimulation observed in selenium-supplemented control CHO cells. Similarly, TR1 activity in the i⁶A⁻ background was 15% of control cells (Fig. 1B, $p < 0.001$), and, as seen with GPx-1, did not increase in

selenium-supplemented media (Fig. 1B). Therefore, the data obtained assessing the levels of these two selenoproteins in the i⁶A⁻ background was consistent with that seen when UGA translation was assessed using the reporter constructs in the same cells.

In contrast to the increase in UGA translation observed when the SECIS-containing reporter constructs were stably transfected into ST4 tRNA^{[Ser]Sec} overexpressing cells (Fig. 5), the activities of both GPx-1 and TR1 did not increase in ST4, there being a marginal, but significant reduction in the activities for both selenoenzymes (Fig. 1A,B). Following selenium supplementation of the media of ST4 cells, both GPx-1 and TR1 levels were induced to the same degree as observed in control cells. The reason for the discrepancy between the effect of elevated tRNA^{[Ser]Sec} levels on UGA translation obtained using the reporter constructs (increase) and GPx-1/TR1 levels (no effect) was further investigated.

Either selenoprotein mRNA or tRNA^{[Ser]Sec} can be limiting for selenoprotein synthesis

SECIS efficiency using the pBPLUGA reporter constructs was increased in CHO cells overexpressing tRNA^{[Ser]Sec}, but neither GPx-1 or TR1 levels increased in the same cells. Because the reporter assay was performed using transient transfection, which typically results in high levels of mRNA from transfected DNA, and CHO cells have relatively low endogenous GPx-1 and TR1 mRNA levels, it is possible that tRNA^{[Ser]Sec} was limiting for translation of the mRNA derived from the reporter construct, but the native GPx-1

FIGURE 5. Elevated tRNA^{[Ser]Sec} increases UGA translational efficiency to the same degree in selenium-supplemented or -unsupplemented cells. Control (pLNCX) transfected CHO cells and CHO cells overexpressing tRNA^{[Ser]Sec} (ST4) were transfected with the pBPLUGA reporter constructs containing SECIS sequences from the five indicated selenoproteins, followed by incubation for 3 d in standard medium or medium supplemented with 30 nM sodium selenite. UGA translation was measured as luciferase activity achieved by the translation of the in-frame UGA codon separating the luc and βgal genes, and normalized by dividing by βgal activity. Values are the average of nine independent transfections ± standard deviation, expressed as the percent increase over UGA translation in control cells incubated in standard medium or selenium-supplemented medium.

FIGURE 6. Reduced levels of the methylated tRNA^{[Ser]Sec} isoform reduce UGA translation efficiency. Control-transfected (pLNCX) CHO cells and cells overexpressing an A37G tRNA^{[Ser]Sec} mutant (i⁶A⁻), were transfected with the pBPLUGA reporter constructs containing SECIS sequences from the five indicated selenoproteins, followed by incubation for 3 d in standard medium or medium supplemented with 30 nM sodium selenite. UGA translation was measured as luciferase activity achieved by the translation of the in-frame UGA codon separating the luc and βgal genes, and normalized by dividing by βgal activity. Values are the average of three independent transfections ± standard deviation.

transcript was not rate limiting in control cells. To test the hypothesis that tRNA^{[Ser]Sec} can be limiting for GPx-1 translation in circumstances of high levels of its mRNA, the overexpression of tRNA^{[Ser]Sec} was achieved in cells previously engineered to overexpress GPx-1 mRNA, and the effects on GPx-1 activity were determined.

CHO transfectants that overexpress GPx-1 mRNA and protein by five- to sevenfold (Mansur et al. 2001), referred to as GPx7, were stably transfected with a DNA fragment encoding the mouse tRNA^{[Ser]Sec} gene, and individual colonies overexpressing tRNA^{[Ser]Sec} by 14- and 25-fold, as determined by Northern blot analysis (data not shown), were selected for further study. A dose-dependent increase of GPx-1 activity with increasing tRNA^{[Ser]Sec} levels was observed (Fig. 7); CHO cells overexpressing tRNA^{[Ser]Sec} by 14- and 25-fold exhibited GPx-1 activity of 170% and 250%, respectively, as compared to control GPx7 cells with native amounts of tRNA^{[Ser]Sec}. These results indicate that tRNA^{[Ser]Sec} could be rate limiting for GPx-1 translation when the corresponding mRNA is present at high levels. Following selenium supplementation, GPx-1 activities were further increased by 140% and 200% for the 14- and 25-fold tRNA^{[Ser]Sec} overproducers as well.

The mcm⁵Um/mcm⁵U ratio in tRNA^{[Ser]Sec}-transfected GPx7 cells was the same as the nontransfected GPx-7s, in both cases each representing approximately 50% of the tRNA^{[Ser]Sec} population, and the relative distribution of mcm⁵Um in both cell lines increased following selenium supplementation to 54% and 71%, respectively (Table 1). Thus, the absolute level of mcm⁵Um was higher following

transfection and subsequent selenium supplementation, consistent with the hypothesis that this isoform is critical for UGA translation.

The role of mcm⁵Um in selenoprotein translation

As part of the studies described in the preceding section, it was observed by Northern blot analysis that total tRNA^{[Ser]Sec} levels rose by 2.4-fold in GPx7, relative to control transfected cells (data not shown). It has been previously documented that tRNA usage results in increased amounts of that isoacceptor, presumably by protecting that molecule from cellular degradation (Maenpaa and Bernfield 1969; Negrutskii and Deutscher 1992). To examine whether the increased translation of GPx-1 mRNA in GPx7 cells resulted in a differential protection of tRNA^{[Ser]Sec} isoforms, the distribution of mcm⁵U and mcm⁵Um was examined in GPx7 cells by RPC-5 chro-

matography. In these cells, the distribution of the two isoforms was shifted toward mcm⁵Um in comparison to control cells, with 51% of total tRNA^{[Ser]Sec} in the methylated form, 2.3-fold greater than the 22% in control cells (Table 1). Following incubation in selenium-supplemented medium (under conditions of translational induction of GPx-1), the level of mcm⁵Um in GPx7 further increased to 71% of total tRNA^{[Ser]Sec}, significantly greater than the relative amount of mcm⁵Um in selenium-supplemented control

FIGURE 7. Elevated tRNA^{[Ser]Sec} increases GPx-1 activity in GPx-1-overexpressing cells. CHO transfectants that overexpress GPx-1 mRNA (GPx7) were stably transfected to overexpress tRNA^{[Ser]Sec} by 14- and 25-fold. Control cells are CHO cells transfected with the pLNCX vector only. Values are the average of three GPx-1 activity determinations measured by a coupled spectrophotometric assay, and activity is expressed as nanomoles of NADPH per minute per milligram of protein GPx-1 activities.

cells. These results are consistent with preferential utilization of mcm^5Um during GPx-1 translation. Were mcm^5Um preferentially used during UGA translation, then overexpression of GPx-1 might reduce UGA translation from the SECIS-containing pBPLUGA constructs by competing for limiting levels of mcm^5Um . To investigate this possibility, SECIS efficiency was assessed in GPx7 cells and found to be reduced for all of the SECIS-containing reporter elements examined (Fig. 8). SECIS-independent UGA suppression in GPx7 similarly declined as compared to control cells ($p < 0.01$), and did not increase with selenium supplementation (Fig. 2).

Time course analyses were also performed to examine the kinetics of the effects of selenium supplementation on GPx-1 translational stimulation and $\text{tRNA}^{[\text{Ser}]\text{Sec}}$ methylation. CHO cells were supplemented with selenium for 5 d and the isoform distribution and GPx-1 activity assessed. The mcm^5Um portion of $\text{tRNA}^{[\text{Ser}]\text{Sec}}$ increased from 22.4% at baseline to a plateau level of 39.6% after 1-d supplementation (Table 1, Control 1d Se; Fig. 9). In contrast, the selenium-mediated induction of GPx-1 levels plateaued at d 3, with a 4.7-fold induction, indicating that events in addition to the methylation of $\text{tRNA}^{[\text{Ser}]\text{Sec}}$ are likely required for maximal GPx-1 induction.

DISCUSSION

The findings presented here contribute to an increasingly complex picture of the selenoprotein synthesis by elaborating on the regulation of Sec incorporation at UGA codons. The use of the pBPLUGA reporter construct permits UGA translation to be examined independently of other influences that might affect selenoprotein levels, and effects of selenium, $\text{tRNA}^{[\text{Ser}]\text{Sec}}$, and selenoprotein mRNA levels were evaluated and compared to the effects of the same

FIGURE 8. Elevated selenoprotein mRNA levels reduce UGA translational efficiency. CHO transfectants that overexpress GPx-1 (GPx7) were transfected with the pBPLUGA reporter constructs containing SECIS sequences from the five indicated selenoproteins. UGA translation was measured as luciferase activity and normalized by dividing by βgal activity. Values are the average of three independent transfections $\pm$ standard deviation.

FIGURE 9. Time course of the increase in mcm^5Um and GPx-1 activity following increased selenium. Control CHO cells were incubated in 30 nM selenium for the number of days indicated followed by determination of GPx-1 activity and $\text{tRNA}^{[\text{Ser}]\text{Sec}}$ isoform distribution. GPx-1 values are the average of three cell lysates $\pm$ standard deviation. The level of the methylated isoform was measured by RPC-5 chromatography, and is expressed as the percentage of the total of both $\text{tRNA}^{[\text{Ser}]\text{Sec}}$ isoforms.

variables on representative selenoprotein levels. These results provide evidence in support of the role of the mcm^5Um isoform in selenoprotein translation and for a possible regulatory role for the Sec-inserting tRNA.

The activity of luc from the pBPLUGA vector without an inserted SECIS element reflects the suppression of in-frame UGA codons independently from the process of Sec incorporation. SECIS-independent UGA stop codon suppression was found to be unaffected by the changes in $\text{tRNA}^{[\text{Ser}]\text{Sec}}$ isoform distribution that accompanied selenium supplementation. In contrast, UGA suppression was elevated in $\text{tRNA}^{[\text{Ser}]\text{Sec}}$ overexpressing cells, ST4. These results are consistent with previous data derived from in vitro examination of UGA suppression in GPx-1 mRNA (Jung et al. 1994). In rabbit reticulocyte lysates programmed with rabbit β -globin mRNA, seryl- $\text{tRNA}^{[\text{Ser}]\text{Sec}}(\text{mcm}^5\text{Um})$ and unaminoacylated $\text{tRNA}^{[\text{Ser}]\text{Sec}}$ both contributed to translation of a full-length peptide. In contrast, neither isoform of $\text{tRNA}^{[\text{Ser}]\text{Sec}}$ aminoacylated with Sec yielded detectable full-length product. These results indicate that Ser- $\text{tRNA}^{[\text{Ser}]\text{Sec}}$ suppresses UGA stop codons with greater efficiency than either isoform of Sec- $\text{tRNA}^{[\text{Ser}]\text{Sec}}$ (Jung et al. 1994). Given these earlier observations, and current findings showing selenium does not stimulate UGA suppression under the same conditions in which it increased $\text{tRNA}^{[\text{Ser}]\text{Sec}}$ methylation, it is likely that increased suppression in ST4 cells was due to elevated levels of seryl- $\text{tRNA}^{[\text{Ser}]\text{Sec}}$.

The SECIS element is central to the assembly of the Sec insertion apparatus, and is a potential site of translational regulation. Several SECIS elements have been examined previously in various cells and reporter systems (Berry et al. 1994; Kollmus et al. 1996; Wingler et al. 1999; Low et al.

2000; Nasim et al. 2000), and this study expands the set examined to include TR3 and the two naturally occurring genetic variants of the Sep15 SECIS. The SECIS elements examined herein were found to exhibit a range of UGA translation efficiencies in CHO cells, from 8- to 50-fold above SECIS-independent suppression in the order of Sep15(TA) > GPx-1 > Sep15(CG) > TR1 = TR3. These differences in baseline activity are therefore a function of the variation in primary structure for each SECIS element. In contrast, the efficiency of the five SECIS elements tested responded to different extents in response to selenium supplementation, increased levels of tRNA^{[Ser]Sec}, and changes in tRNA^{[Ser]Sec} isoform distribution. The data presented here indicates that the SECIS element may contribute to the individual responses of selenoproteins under conditions of changing selenium availability and the consequential biochemical changes.

The methylation of mcm⁵U to mcm⁵Um is likely to be an important event in the regulation of selenoprotein synthesis, yet functional differences between these isoforms have not yet been identified. Several lines of evidence support the possibility that mcm⁵Um is preferentially used in selenoprotein translation. Selenium supplementation typically results in a relative increase in mcm⁵Um levels as well as selenoprotein synthesis (Hatfield et al. 1991; Diamond et al. 1993). Of note was the observation that when three different human glioma cell lines were examined for effects of selenium on GPx-1 induction and tRNA^{[Ser]Sec} isoform distribution, the degree of methylation was correlated with the magnitude of GPx-1 induction (Mansur et al. 2000). Using the pBPLUGA reporter construct, it was determined that the effects of selenium supplementation, at least in part, are likely due to increased translation of the UGA codon as Sec. In addition, reducing the levels of mcm⁵Um by transfection of a dominantly acting mutant tRNA resulted in the attenuation of UGA translation, consistent with a role of the methylated isoform in selenoprotein translation. Additional data in support of the role of mcm⁵Um was obtained by the observation that its steady-state levels increase with GPx-1 overexpression. In light of previous studies showing that turnover of tRNA is reduced by increased utilization in protein synthesis (Kanerva and Maenpää 1981) and that the turnover of mcm⁵Um may be slowed by selenium supplementation (Jameson et al. 2002), the elevation of mcm⁵Um levels in GPx7 cells is consistent with preferential utilization of mcm⁵Um in the translation of selenoproteins. Time course data presented in Figure 9 indicated that both tRNA methylation and GPx-1 levels rise with selenium supplementation, and that the stimulation of methylation reached a plateau by d 1, with GPx-1 induction reaching plateau at d 3. These results indicate that although an increase in GPx-1 activity is concurrent with the rise in the methylated isoform, other events resulting from increased selenium may contribute to the rise in selenoprotein levels. The identification and manipulation of the enzyme that methylates tRNA^{[Ser]Sec}

in response to selenium will permit the direct determination of role of mcm⁵Um in selenoprotein translation.

Elevated tRNA^{[Ser]Sec} levels increased UGA translation in the transient reporter assay, under conditions of high levels of UGA-containing template mRNA, but did not increase the activity of endogenous GPx-1 and TR1 in CHO cells. It is therefore likely that tRNA^{[Ser]Sec} is limiting for UGA translation in the transient assay, but not for selenoprotein synthesis in cells with much lower levels of selenoprotein mRNA. This suggestion is supported by the dose-dependent increase in GPx-1 activity with increasing tRNA^{[Ser]Sec} levels in cells with high GPx-1 mRNA levels (Fig. 7) and the observation that GPx-1 overexpression resulted in reduced UGA translation from all of the tested reporter constructs (Fig. 8). It is therefore suggested that in tissues such as the liver, with high levels of selenoprotein mRNA, (Bermano et al. 1995) and protein (Chittum et al. 1997; Hornberger et al. 2003), translation may be influenced by the level of tRNA^{[Ser]Sec} and provide an additional example of a possible regulatory role of tRNA^{[Ser]Sec} in selenoprotein synthesis.

MATERIALS AND METHODS

Cell culture

CHO cells were grown in α -MEM (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Bio-Wittaker). Where indicated, cells were incubated in medium supplemented with an aqueous solution of Na₂SeO₃ to a final concentration of 30 nM. Stable and transient transfections were carried out with Lipofectin (Life Technologies), following the manufacturer's protocol. Stable transfections were performed in 60-mm plates of cells at approximately 80% confluence, transfected with a mixture containing 2 μ g DNA with 12 μ L Lipofectin for 6 h in serum-free media, and then incubated in standard medium for 3 d. Transfectants were subsequently selected in geneticin G418 (Sigma), 50 μ g/mL, or Zeocin (Invitrogen) at 300 μ g/mL and colonies were recovered and expanded for further analysis. Transient transfections were performed in 35-mm wells with cells at 30% confluence, using 0.7 μ g of DNA and 4 μ L Lipofectin per well for 6 h, and the media was replaced as indicated in the text.

Overexpression of tRNA^{[Ser]Sec} in CHO cells was achieved by cotransfection of 2 μ g of a 1.93 Kbp XhoI-StuI fragment encoding the wild-type or A37G mutant mouse tRNA^{[Ser]Sec} gene (Ohama et al. 1994) and 0.25 μ g of pLNCX with the Lipofectin reagent, as described above. Overexpression of tRNA^{[Ser]Sec} in GPx-1 overexpressing cells was achieved by cotransfection of the 1.93-Kbp fragment with 2 μ g pcDNA3.1/Zeo/Cat vector (Life Sciences).

DNA constructs

A series of constructs was generated in which the 3' untranslated region, including the SECIS element, of several selenoproteins was inserted into the pBPLUGA reporter construct (Kollmus et al. 1996). These DNA segments were obtained by PCR and subsequent ligation into the pBPLUGA polycloning site. The primers used for PCR amplification, the template DNA source, and inser-

tion site are described in Table 2. Following transformation of DH5 α competent bacteria, clones were isolated, screened by restriction endonuclease digestion, and the identity of the insert was confirmed by DNA sequencing.

Reporter assay

SECIS efficiency was determined by measuring luciferase (luc) and β -galactosidase (β gal) activity in extracts obtained from transiently transfected cells. Following transfection with the reporter constructs described above, cells were incubated for 3 d in control or selenium-supplemented medium and subsequently lysed with Reporter Lysis Buffer (Promega), and the insoluble fraction collected by centrifugation at 14,000g for 2 min. Luciferase activity was determined with the Luciferase 1000 Assay System (Promega) and a Zylux FB12 luminometer. The β -Galactosidase Enzyme Assay System (Promega) was used for 250- μ L reaction volume in 96-well plates, read at 420 nm in a StatFax plate reader (Awareness Technology). Reactions for each lysate were performed in duplicate and determined to be in a linear range of activity for a standard curve. UGA translation efficiency was described as the relative luminometer units divided by β gal activity (A_{420}), to normalize for transfection efficiency.

Northern blot analysis

Total tRNA was extracted from CHO cells using DEAE cellulose and quantified by absorbance at 260 nm as described (Roe 1975). Five μ g of tRNA per sample were electrophoresed on 15% polyacrylamide/4 M urea gel (acrylamide:bis-acrylamide, 30:1) and transferred by capillary action to Gene Screen Plus membranes (Perkin Elmer) according to the product guidelines. Filters were hybridized with 32 P-labeled probes representing either tRNA^{[Ser]^{Sec}} or tRNA^{Ser}. A probe for tRNA^{[Ser]^{Sec}} was generated by random primer labeling (Feinberg and Vogelstein 1983) from a 193-nt template containing the human tRNA^{[Ser]^{Sec}} gene (O'Neill et al. 1985). Double-stranded complementary tRNA^{Ser} (GenBank accession number M38616) was generated by annealing two complementary oligonucleotides, one of which corresponded to 24 nt of the 5' end of tRNA^{Ser} with three additional non-base-pairing guanosines at the 5' end: (5'-GGGCAGGTTCTGAATCCTGCC GACTACG-3') and a second oligonucleotide, complementary to

the first (5'-CGTAGTCGGCAGGATTCTGAACCTG-3'). A hybridization probe was generated by annealing the oligonucleotides and the extension of the protruding 3' end with Klenow fragment (Boehringer Mannheim), 80 μ Ci α - 32 P-dCTP (Perkin Elmer, 3000 Ci/mmol), and 200 μ moles/L each dATP, dGTP, dTTP (Invitrogen). Hybridization was performed as described by the manufacturers' recommendations. Washing conditions for the tRNA^{[Ser]^{Sec}} hybridization were 0.1 $\times$ SSC, 0.1% SDS for 3 h at 65°C. Wash conditions for the tRNA^{Ser} hybridization on Gene Screen Plus were two 1-h washes in 2 $\times$ SSC, 0.1% SDS at 55°C. Bands visualized in autoradiograms were quantified with a Bio-Rad GS-710 laser densitometer.

RPC-5 chromatography

The distribution of tRNA^{[Ser]^{Sec}} isoforms was determined by RPC-5 chromatography. Total tRNA from approximately 1 g of CHO cells was deacylated, aminoacylated with [3 H]serine, which labels both serine and Sec tRNAs, and chromatographed on an RPC-5 column (Kelmers and Heatherly 1971) as described previously (Moustafa et al. 1998; Carlson and Hatfield 2002). The aminoacylated tRNA was chromatographed twice on the RPC-5 column, first in the absence and then presence of Mg²⁺, as described (Hatfield et al. 1979). These two chromatographic steps resolve seryl-tRNA^{[Ser]^{Sec}} from seryl-tRNA^{Ser} and allow the quantification of the tRNA^{[Ser]^{Sec}} population relative to the total seryl-tRNA population.

Selenoenzyme activity assays

GPx-1 activity was determined by a coupled spectrophotometric assay that measures GPx activity coupled to the oxidation of NADPH as described (Samuels et al. 1991). Briefly, sonicated cell extracts are incubated with glutathione reductase, hydrogen peroxide, sodium azide, NADPH, and glutathione. The change in absorbance, measured at 339 nm, was monitored at 0.5-min intervals for 5 min. The protein content of the cell extract was measured by Bradford assay (BioRad). The assay results are expressed as nanomoles NADPH oxidized per minute per milligram of cell protein.

The activity of TR was measured optically through the reduction of sulfhydryl groups by a timed reaction with recombinant

TABLE 2. Constructs containing SECIS elements in the pBPLUGA reporter vector

Selenoprotein	Primers	Positions of cDNA amplified	DNA source	pBPLUGA polylinker sites
GPx-1	5'-AACTAGTACCATGACATCGAGCCTG-3' 5'-AACTGCAGCAGAGACTGGGATCAACA-3'	587–810	hGPx-1	SpeI PstI
TR1	5'-AAAAAGATCTCCATCGGCATCCACCC-3' 5'-AAAGGAAAGCGCGCGTTGAGCTCAACAGAC-3'	1814–2225	ATCC #5958600	BglII
TR3	5'-AAAAAGATCTGCCTGCATTTCCTTGGC-3' 5'-ATATAATAGCGGCGCTTGCAGAAATGCCA-3'	1278–1790	Murine DNA fragment ResGen #2820260	NotI
Sep15(GC)	5'-AACTAGTGCTTGTAAACAGACTTGCAGTTA ATTATGC-3'	733–1216	Mammalian gene collection (CG) 1535CPTX (Bright et al. 1997)	SpeI PstI
Sep15(TA)	5'-AACTGCAGGGTCTTACAAATGATCACTTTTA AATGGAC-3'		(TA) STSAS90 (Samuels et al. 1991)	

Escherichia coli thioredoxin (Calbiochem), insulin (Sigma), and NADPH (Sigma; Arner et al. 1999). Cell lysates were partially purified by affinity chromatography on a 2'5'-ADP-sepharose (Pharmacia) column. The reaction was terminated by the addition of DNTB and Guanidine HCl, and the optical density of the reactions at 412 nm was compared to a standard curve obtained with recombinant *E. coli* TR (Sigma).

Statistics

Data obtained with the reporter constructs were compared by two-way ANOVA. A two-tailed *t* test was used to compare enzyme activities.

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