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Selective Rescue of Selenoprotein Expression in Mice Lacking a Highly Specialized Methyl Group in Selenocysteine tRNA*

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Selenocysteine (Sec) is the 21st amino acid in the genetic code. Its tRNA is variably methylated on the 2′-*O*-hydroxyl site of the ribosyl moiety at position 34 (Um34). Herein, we identified a role of Um34 in regulating the expression of some, but not all, selenoproteins. A strain of knock-out transgenic mice was generated, wherein the Sec tRNA gene was replaced with either wild type or mutant Sec tRNA transgenes. The mutant transgene yielded a tRNA that lacked two base modifications, N^6-isopentenyladenosine at position 37 (i^6A37) and Um34. Several selenoproteins, including glutathione peroxidases 1 and 3, SelR, and SelT, were not detected in mice rescued with the mutant transgene, whereas other selenoproteins, including thioredoxin reductases 1 and 3 and glutathione peroxidase 4, were expressed in normal or reduced levels. Northern blot analysis suggested that other selenoproteins (*e.g.* SelW) were also poorly expressed. This novel regulation of protein expression occurred at the level of translation and manifested a tissue-specific pattern. The available data suggest that the Um34 modification has greater influence than the i^6A37 modification in regulating the expression of various mammalian selenoproteins and Um34 is required for synthesis of several members of this protein class. Many proteins that were poorly rescued appear to be involved in responses to stress, and their expression is also highly dependent on selenium in the diet. Furthermore, their mRNA levels are regulated by selenium and are subject to nonsense-mediated decay. Overall, this study described a novel mechanism of regulation of protein expression by tRNA modification that is in turn regulated by levels of the trace element, selenium.

The mechanism of selenocysteine (Sec)[1] incorporation into protein as the 21st amino acid was elucidated in *Escherichia coli* by Böck (reviewed in Ref. 1). In mammals, the mechanism of Sec insertion into protein is not as completely understood (reviewed in Refs. 2 and 3). However, both prokaryotes and eukaryotes use the stop codon, UGA, to dictate the incorporation of Sec after the tRNA is initially aminoacylated with serine, and the biosynthesis of Sec occurs on its tRNA. The tRNA has therefore been designated Sec $tRNA^{[Ser]Sec}$ (2). The presence of a stem-loop structure that occurs downstream of UGA in selenoprotein mRNA, known as a Sec insertion sequence (SECIS) element (4), is responsible for dictating UGA as Sec instead of the cessation of protein synthesis. In mammals, a specific SECIS-binding protein, designated SECIS-binding protein 2 (5), recognizes the SECIS element, and a specific elongation factor, designated EFsec (6, 7), recognizes selenocysteyl-$tRNA^{[Ser]Sec}$, and the resulting complex guides Sec into the nascent polypeptide in response to UGA (2, 3).

In higher vertebrates, there are two Sec $tRNA^{[Ser]Sec}$ isoforms that differ from each other by a single nucleoside modification at position 34, which is the wobble position of the anticodon (2). One isoform contains methylcarboxylmethyluridine (mcm^5U) at this site, and the other contains methylcarboxymethyluridine-2′-*O*-methylribose (mcm^5Um). Several lines of evidence suggest that methylation of the ribosyl moiety at the 2′-O-hydroxyl site (designated Um34) is a highly specialized event. It is the last step in the maturation of Sec $tRNA^{[Ser]Sec}$. This modification step is dependent on the prior synthesis of the four modified bases found in Sec $tRNA^{[Ser]Sec}$ and on an intact tertiary structure, whereas synthesis of the other modified nucleosides, including mcm^5U, is less stringently connected to primary and tertiary structure (8). The methylation step is influenced by selenium status, whereby the levels of mcm^5U are enriched and mcm^5Um reduced under conditions of selenium deficiency, and the ratio of the two isoforms is reversed under conditions of selenium sufficiency (2). The presence of Um34 dramatically affects Sec $tRNA^{[Ser]Sec}$ secondary and tertiary structure (9). The presence of Um34 on Sec $tRNA^{[Ser]Sec}$ correlated with the expression of certain selenoproteins (*e.g.* GPx1) (10, 11), whereas enrichment of the isoform lacking Um34 correlated with the expression of other selenoproteins (*e.g.* TR3) (11). In addition, a specialized role of the selenium-induced, Um34 $tRNA^{[Ser]Sec}$ in selenoprotein translation was recently reported (12). However, the specific role of this isoform in selenoprotein synthesis has not been elucidated.

The selenoprotein population in rodents is composed of 24 members, and there are 25 members in humans (13). The function of many of these selenoproteins is not known. However, several approaches have been used in assessing their function and to provide better insights into their possible roles in health. Direct assays of selenoproteins such as glutathione peroxidase 1 (GPx1) and thioredoxin reductase 1 (TR1) demonstrate that they can function as antioxidants (14, 15), whereas genetic and biochemical characterization of selenoproteins such as Sep15 (16, 17) and GPx1 (18, 19) suggests that these members may in addition have roles in cancer prevention.

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[1] The abbreviations used are: Sec, selenocysteine; SECIS, Sec insertion sequence; mcm^5U, methylcarboxylmethyluridine; mcm^5Um, methylcarboxymethyluridine-2′-*O*-methylribose; GPx1, -2, and -4, glutathione peroxidase 1, 2, and 4, respectively; TR1 and -3, thioredoxin reductase, 1 and 3, respectively; SelP, selenoprotein P; i^6A37, N^6-isopentenyladenosine at position 37.

Gene knock-out studies involving selenoproteins in mice show that some, such as glutathione peroxidase 4 (GPx4) (20), selenoprotein P (SelP) (21, 22), thyroid hormone deiodinase 2 (23), and mitochondrial thioredoxin reductase (TR3) (24) have essential roles in cellular function, since their removal is lethal or results in an abnormal phenotypic change. Other selenoproteins, such as GPx1 (25) and glutathione peroxidase 2 (GPx2) (26), probably have nonessential roles, since their removal manifests little or no phenotypic change. Exposing animals lacking a nonessential selenoprotein to stress, however, demonstrates that the animal may not cope with certain stresses compared with their wild type counterparts (27, 28). Thus, some selenoproteins that are nonessential to life of the organism probably provide protection from environmental stress.

Several studies have examined the effect of altering Sec tRNA$^{[Ser]Sec}$ expression on selenoprotein biosynthesis, which provided another means of elucidating the function of selenoproteins and their roles in health. Removal of *trsp* in knock-out mice is embryonic lethal, demonstrating that selenoproteins are essential to mammalian development (29, 30). Generation of a conditional knock-out of *trsp* using *loxP-Cre* technology has shown that Sec tRNA$^{[Ser]Sec}$ levels can be reduced in mammary tissue by about 80%, resulting in an altered selenoprotein expression in a selenoprotein-specific manner (30), whereas complete removal of *trsp* in liver demonstrated that selenoprotein expression is required for proper function of this organ (31). Overexpression of Sec tRNA$^{[Ser]Sec}$ in transgenic mice carrying extra copies of the wild type transgene resulted in little or no effect on selenoprotein expression (11). However, transgenic mice carrying extra copies of a Sec tRNA$^{[Ser]Sec}$ mutant transgene that produces a tRNA gene product lacking N^6-isopentenyladenosine (i^{6}A37) at this site and Um34 (8) affect selenoprotein synthesis in a selenoprotein- and tissue-specific manner (11).

In the present study, transgenic mice possessing *trsp* wild type or mutant transgenes (11) were used to rescue *trsp* null mice (30). Mice dependent on the wild type transgene for survival manifested little or no change in selenoprotein expression, whereas mice dependent on the i^{6}A mutant transgene rescued only some selenoproteins. Rescued selenoproteins included TR1 and TR3, whereas those that were poorly rescued included GPx1, GPx3, SelR, SelT, and SelW. Other selenoproteins, such as GPx2, GPx4, SelP, and Sep15, appeared to be partially rescued. These studies not only show that expression of some selenoproteins in mammals is highly dependent on Um34, but generating genetically altered mice that express reduced levels of a particular subclass of selenoproteins provides a unique model for examining the function of certain selenoproteins and their possible roles in health.

EXPERIMENTAL PROCEDURES

Materials and Animals—Selenium-75 (specific activity 1000 Ci/mmol) was obtained from the Research Reactor Facility, University of Missouri (Columbia, MO); [α-^{32}P]dCTP (specific activity ~6000 Ci/mmol) was from PerkinElmer Life Sciences; and [^{3}H]serine (specific activity 29 Ci/mmol) and Hybond Nylon N$^+$ membranes were from Amersham Biosciences. NuPage 10% polyacrylamide gels, polyvinylidene difluoride membranes, Superscript II reverse transcriptase, See-Blue Plus2-protein markers, and the expressed sequence tag clone used in making the GPx2 probe (IMAGE Consortium Clone ID 6395265 (AN BQ961211)) were purchased from Invitrogen, and Universal Reference RNA was from Stratagene. SuperSignal West Dura extended duration substrate was obtained from Pierce, PCI-Neo vector was from Promega, and anti-rabbit horseradish peroxidase- and anti-chicken horseradish peroxidase-conjugated secondary antibodies were obtained from Sigma. All other reagents were obtained commercially and were products of the highest grade available. Mice (strain C57BL/6) that were heterozygous for the Sec tRNA$^{[Ser]Sec}$ gene (wild type gene is designated as *trsp* and the deleted gene as Δ*trsp*) were from a previous study in our laboratory (30), as were the transgenic mice that were either homozygous for the wild type transgenes (strain FVB/N) wherein each allele carried 10 copies of the Sec tRNA$^{[Ser]Sec}$ transgene (designated *trsp^t*) or for the mutant Sec tRNA$^{[Ser]Sec}$ transgenes whose product lacked i^{6}A37 and Um34, wherein each allele carried 20 copies of the mutant transgene (designated *trsp^t*i6A$^-$) (11). Antibodies against GPx1 were obtained from Qichang Shen, antibodies against GPx2 were from Regina Brigelius-Flohé, and antibodies against GPx4 were from Donna Driscoll, whereas antibodies against TR1, TR3, SelR, SelT, and Sep15 were from our laboratories (13, 32).[2] The care of animals was in accordance with the National Institutes of Health institutional guidelines under the expert direction of Dr. Kyle Stump (NCI, National Institutes of Health, Bethesda, MD).

Genotyping Mice—DNA was extracted from mouse tail clippings and the presence or absence of *trsp*, *trsp^t*, and *trsp^t*i^{6}A$^-$ determined by PCR with the appropriate primers. The forward and reverse primers designated 5FPROA and CKNOR12, respectively, that complement bases beginning at −2137 bp and −255 bp upstream of the gene (30, 31) were used to detect *trsp* (yielding a 1.9-kb PCR fragment). CKNO2, a forward primer that complements bases beginning at −442 bp upstream of *trsp* (30), was used to detect *trsp*. Two new reverse primers, designated RES1 (5′-ccttgtgagacgaccttctatg-3′) and VP1 (5′-tgtggaattgtgagcggata-3′), where RES1 complements bases beginning at +538 bp downstream of *trsp* (the 5′-end of *trsp* is base +1) and VP1 corresponds to that portion of the vector sequence (pBluescript II) retained in the transgene for monitoring its insertion into the genome (11), were used to monitor, along with the forward primer, CKNO2, the presence or loss of *trsp* (CKNO2-RES1) or *trsp^t* and *trsp^t*i^{6}A$^-$ (CKNO2-VP1). CKNO2-RES1 yields a 980-bp PCR *trsp* fragment and a 500-bp PCR Δ*trsp* fragment, and CKNO2-VP1 yields a 1072-bp PCR *trsp^t* or *trsp^t*i^{6}A$^-$ fragment.

Isolation, Aminoacylation, Fractionation, and Quantification of tRNA and Primer Extension—Total tRNA was isolated from mouse liver, aminoacylated with [^{3}H]serine and 19 unlabeled amino acids in the presence of rabbit reticulocyte synthetases (33), the resulting aminoacylated tRNA fractionated on a RPC-5 column (34) in the absence and subsequently in the presence of Mg^{2+} as given (11, 30). The amount of Sec tRNA$^{[Ser]Sec}$ expressed from the host *trsp* or from wild type (*trsp^t*) or mutant (*trsp^t*i^{6}A$^-$) transgenes relative to the total Ser tRNA population and the distributions of mcm^{5}U and mcm^{5}Um has been detailed elsewhere (11, 30). The presence of U at position 9 in host Sec tRNA$^{[Ser]Sec}$ and of a C at position 9 in the transgene Sec tRNA$^{[Ser]Sec}$ provided a means of distinguishing host from transgene-generated tRNA$^{[Ser]Sec}$ by primer extension using either ddG or ddA in the reaction and the appropriate primer as given (see Ref. 11 and references therein).

Labeling of Selenoproteins and GPx Activity Assays and Selenium Assay—Mice were injected intraperitoneally with 50 μCi of ^{75}Se/g and sacrificed 48 h after injection. Tissues and organs were excised, immediately frozen in liquid nitrogen and stored at −80 °C until ready for use. Tissues were homogenized, the extracts were electrophoresed along with molecular weight markers, and the developed gel was stained with Coomassie Blue, dried, and exposed to a PhosphorImager as described (see Ref. 31 and references therein).

GPx1–3 activities were measured using a standard assay with hydrogen peroxide as substrate as described previously (11, 30, 31). The amount of selenium in extracts of liver, heart, brain, and testes was determined by Oscar E. Olsen Biochemistry Laboratories at South Dakota State University as described (31).

Northern and Western Blot Analyses—Total RNA was isolated from liver, kidney, intestine, brain and testes, quantified and loaded onto gels, and transblotted onto a nylon membrane; the membrane was hybridized with ^{32}P-labeled probe; and the Northern blot was analyzed with a PhosphorImager as given (11). GPx1, GPx4, SPS2, D1, TR1, and SelP probes were prepared and used as described (11). The SelV 733-bp probe was obtained from James Weaver, who generated it by digestion of a mouse SelV 1.16-bp XhoI-NotI fragment with BamHI. The remaining probes were generated by RT-PCR using Superscript II reverse transcriptase and universal reference RNA or mouse liver RNA (11) with the exception of GPx2, which was generated from an expressed sequence tag clone (see "Materials and Animals"). Sizes of all RT-PCR-generated probes were in the 500–1200-bp range.

Protein extracts were prepared from liver, kidney, brain, testes, and intestine, electrophoresed on NuPage 10% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and immunoblotted as given (11, 30, 31) with antibodies against Gpx1 (1:1000 dilution), Gpx2

[2] B. A. Carlson, X.-M. Xu, V. N. Gladyshev, and D. L. Hatfield, unpublished observations.

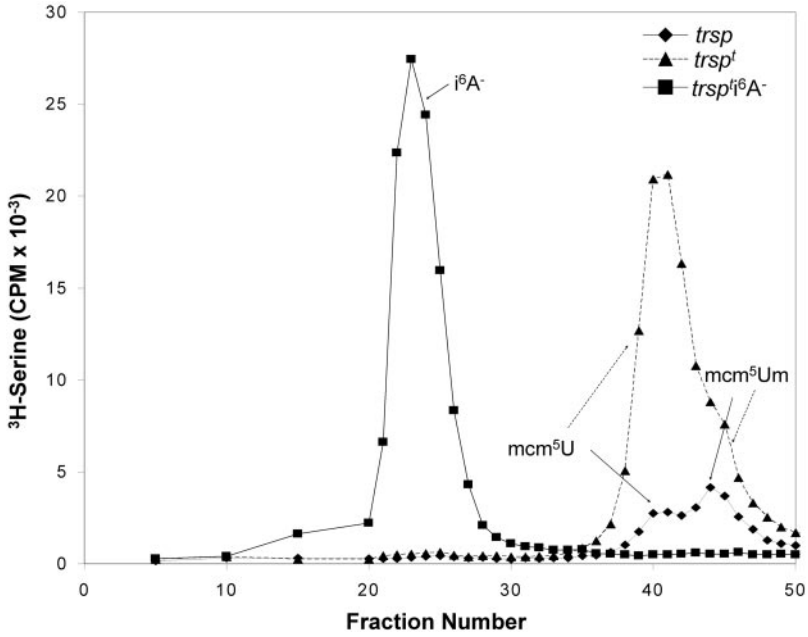

FIG. 1. **Sec tRNA$^{[Ser]Sec}$ levels.** Transfer RNA was isolated from liver of *trsp*, *trsp^t*, and *trsp^t*i^{6}A$^-$ mice, aminoacylated with [^{3}H]serine, and fractionated on an RPC-5 column as described under "Experimental Procedures." A single isoform was present in liver of *trsp^t*i^{6}A$^-$ mice and both mcm^{5}U and mcm^{5}Um isoforms were present in liver of *trsp* and *trsp^t* mice.

(1:1000), Gpx4 (1:2000), SelR (1:1000), SelT (1:400), Sep15 (1:1000), TR1 (1:1000), and TR3 (1:1000). Anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:30,000) was used in all Western blots with the exception of that with TR3, in which anti-chicken horseradish peroxidase-conjugated secondary antibody (1:10,000) was used. Following the attachment of the secondary antibody, membranes were washed with 0.1% TBS-T, incubated in SuperSignal West Dura Extended Duration Substrate and exposed to x-ray film.

RESULTS

Generation of Rescued Mice—To determine the role of the mutant Sec tRNA$^{[Ser]Sec}$, mice were generated wherein the wild type Sec tRNA$^{[Ser]Sec}$ gene (*trsp*) was replaced with several copies of the mutant transgene (*trsp^t*i^{6}A$^-$), and consequently the animal's dependence on *trsp* for survival was replaced with a dependence on *trsp^t*i^{6}A$^-$. This mouse carried 20 copies of a mutant *trsp* transgene (*trsp^t*i^{6}A$^-$) and synthesized elevated amounts of a single form of Sec tRNA$^{[Ser]Sec}$ that lacked i^{6}A37 and, importantly, Um34 (8, 11). The control mouse encoded 20 copies of the wild type transgene (*trsp^t*) and synthesized elevated amounts of wild type Sec tRNA$^{[Ser]Sec}$. These knock-out transgenic mice were designated as rescued mice. To maintain rescued mice with the same number of wild type or mutant transgenes, matings were carried out in the following manner. Mice in which selenoprotein expression was rescued with the mutant transgene were generated by mating parents wherein one parent contained the genotype *trsp*/Δ*trsp-trsp^t*i^{6}A$^-$/*trsp^t*i^{6}A$^-$, and the other parent contained the genotype *trsp*/Δ*trsp-trsp^t*i6A$^{-/+}$. Offspring with genotype Δ*trsp*/Δ*trsp-trsp^t*i^{6}A$^{-/+}$ were selected and used for analysis. Mice in which selenoprotein expression was rescued with the wild type transgene were generated by mating parents wherein both parental strains contained the genotype *trsp*/Δ*trsp-trsp^t*/*trsp^t* and selecting for offspring with genotype Δ*trsp*/Δ*trsp-trsp^t*/*trsp^t*. Mice that were homozygous Δ*trsp-trsp^t* were mated to maintain a stable breeding population. Wild type, control mice (genotype *trsp*/*trsp*; designated *trsp*) were obtained from breedings of parents with the genotype *trsp*/Δ*trsp-trsp^t*/*trsp*$^+$. Genotypes of the rescued mice were determined as described under "Experimental Procedures."

Sec tRNA$^{[Ser]Sec}$ Expression—Transfer RNA was isolated from the livers of mice with genotype *trsp*, Δ*trsp-trsp^t* (hereafter designated *trsp^t*) and Δ*trsp-trsp^t*i^{6}A$^-$ (hereafter designated *trsp^t*-i^{6}A$^-$) and the Sec tRNA$^{[Ser]Sec}$ population analyzed by RPC-5 chromatography (Fig. 1). The Sec tRNA$^{[Ser]Sec}$ population in liver of *trsp* mice consisted of two isoforms. The earlier eluting tRNA contained mcm^{5}U, and the latter eluting form contained mcm^{5}Um (see Refs. 11 and 30 and references therein). The distribution of the two isoforms and the percentage of the Sec tRNA$^{[Ser]Sec}$ population relative to the total seryl-tRNA population were consistent with previous data for the Sec tRNA$^{[Ser]Sec}$ population in liver of *trsp* mice (see Refs. 11 and 30 and references therein). The distributions of the isoforms synthesized from *trsp^t*, which contained much more of the mcm^{5}U isoform (Fig. 1), were similar to those observed in previous studies employing transgenic mice carrying 20 copies of *trsp^t* (11). Since the wild type transgene carries a pyrimidine transition at position 9 (U → C), we confirmed that this mouse synthesized only Sec tRNA$^{[Ser]Sec}$ from *trsp^t* by primer extension (data not shown). The i^{6}A$^-$ isoform is less hydrophobic than its i^{6}A37-containing counterparts, and it elutes much earlier from the column (11). There was no evidence of synthesis of wild type Sec tRNA$^{[Ser]Sec}$ nor of the expression of any other modified isoform of the i^{6}A-deficient species in mice carrying the i^{6}A$^-$ mutant transgene (see also Ref. 8). Furthermore, Um34 cannot be synthesized in i^{6}A$^-$ tRNA$^{[Ser]Sec}$ (8), and the peak of Sec tRNA$^{[Ser]Sec}$ from liver of mice with genotype *trsp^t*i^{6}A$^-$ shown in Fig. 1 consists of a single isoform lacking this methyl modification (11, 30). The amount of this isoform relative to the seryl-tRNA population in liver was consistent with that observed previously in mice carrying the same number of *trsp^t*i^{6}A$^-$ transgenes (11). Overall, these data established that only the mutant transgene lacking i^{6}A37 and Um34 was expressed in mice with genotype *trsp^t*i^{6}A$^-$, whereas a mixture of the wild type Sec tRNA$^{[Ser]Sec}$ forms that were either methylated or unmethylated on the ribosyl moiety at position 34 were expressed in mice with genotype *trsp^t*. Thus, the lack of i^{6}A37 and Um34 in mice carrying *trsp^t*i^{6}A$^-$ did not result in lethality.

^{75}Se Labeling—The expression of selenoproteins in several tissues of mice with genotype *trsp*, *trsp^t*, or *trsp^t*i^{6}A$^-$ was analyzed by labeling animals with ^{75}Se and examining the resulting labeled proteins following gel electrophoresis. Coomassie Blue-stained gels of total proteins from each tissue were compared with their same-tissue counterparts and found to be similar (Fig. 2), suggesting that mice encoding null *trsp* and

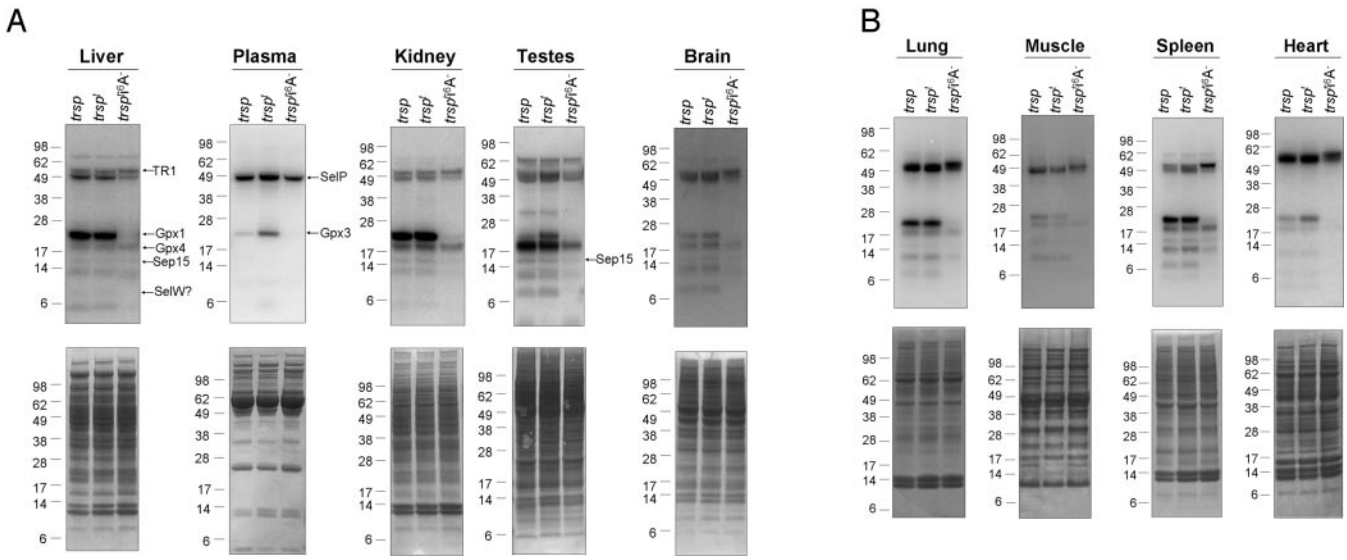

FIG. 2. **^{75}Se-Labeling and selenoprotein analysis.** Mice were labeled with ^{75}Se, and protein was extracted from the tissues in *A* and *B* as shown, electrophoresed, and stained with Coomassie Blue to detect total protein from tissues of *trsp*, *trsp^t*, and *trsp^t*i^{6}A$^-$ mice (see *lower panels* in each tissue) and ^{75}Se-labeled proteins detected from the same tissues with a PhosphorImager (see *upper panels* in each tissue) as described under "Experimental Procedures." Protein marker sizes are shown on the *left* of the *panels* as indicated by the *arrows*, and identified selenoproteins are shown on the *right* of the *panels*.

rescued with *trsp^t* or *trsp^t*i^{6}A$^-$ did not exhibit major differences in protein synthesis as a whole (see also Refs. 11, 30, 31). Examination of ^{75}Se-labeled selenoproteins, however, showed differences in the patterns of labeling within the same tissues of wild type and rescued mice. These differences were particularly apparent between tissues from mice with genotype *trsp* and *trsp^t*i^{6}A$^-$ and between mice with genotype *trsp^t* and *trsp^t*i^{6}A$^-$. TR1, GPx1, GPx4, and Sep15 have been characterized previously in mammalian cells in culture (35) and in mammalian tissues (11, 31). The location of these selenoproteins is designated on the *liver panel* in Fig. 2, and that of Sep15 is also designated on the *testes panel*. SelP and GPx3 have been characterized in plasma (see Ref. 31 and references therein) and are designated on the *plasma panel* in Fig. 2.

Dramatic differences in selenoprotein labeling patterns were observed between mice carrying *trsp* (both wild type and those carrying *trsp^t*) and mice with genotype *trsp^t*i^{6}A$^-$. GPx1 was poorly rescued and was essentially undetectable in the tissues examined, and GPx3 was absent in plasma. Expression of two other selenoproteins was significantly reduced as observed from ^{75}Se labeling. The ^{75}Se-labeled band at approximately ~9 kDa (most likely SelW, based on its size (13)) in liver, testis, lung, and spleen is absent in mice carrying *trsp^t*i^{6}A$^-$. It was designated as SelW with a *question mark* in the *liver panel*. The band at ~32 kDa was absent in testes of mice with genotype *trsp^t*i^{6}A$^-$, and this band may be the nuclear form of GPx4 (see "Western Blot Analyses"). Other selenoproteins such as GPx4 in liver, kidney, testes, lung, and heart appeared to be only partially rescued in mice with genotype *trsp^t*i^{6}A$^-$. The band that migrated at ~49 kDa and just below TR1 may be SelP and/or SPS2 based on their molecular size (13). This band was reduced in liver, kidney, and spleen of mice with genotype *trsp^t*i^{6}A$^-$. In tissues such as heart and lung, this band appeared to be reduced, whereas the slightly slower migrating band (presumably TR1) appeared to be enriched in mice with genotype *trsp^t*i^{6}A$^-$ (see also this latter band in spleen). The identity of the band at ~49 kDa and the possible enrichment of TR1 in these tissues was not further examined.

The selenoprotein labeling patterns in tissues from *trsp* and *trsp^t* mice were very similar in most cases, with the possible exceptions of testes and heart, where GPx1 appeared to enriched, and plasma, where GPx3 appeared to be enriched in the rescued mouse carrying *trsp^t*.

Western Blot Analyses—Rescue of selenoproteins was also examined by Western blotting (Fig. 3). Further confirmation that GPx1 was virtually absent in mice carrying *trsp^t*i^{6}A$^-$, as observed in the ^{75}Se-labeling patterns, was obtained by examining GPx1 levels in liver and kidney by Western analysis. Clearly, GPx1 was not expressed in mice carrying *trsp^t*i^{6}A$^-$. SelR and SelT were also not rescued, whereas GPx2, GPx4, and Sep15 appeared to be only partially rescued in mice carrying *trsp^t*i^{6}A$^-$. The Western blot of GPx4 in testis was overexposed in order that the slower migrating nuclear GPx4 could be visualized. It was not rescued in mice carrying *trsp^t*i^{6}A$^-$.

Other selenoproteins examined by Western analysis showed that TR1 and TR3 were expressed in normal amounts in liver, kidney, and brain of *trsp^t*i^{6}A$^-$ mice.

Taken together, the Western data established that the expression of several selenoproteins was inhibited, whereas that of several others was not affected by replacing *trsp* with *trsp^t*i^{6}A$^-$.

Northern Blot Analyses—The levels of several mRNAs, GPx1, GPx2, GPx3, SelR, SelT, and SelW, were reduced in mice carrying *trsp^t*i^{6}A$^-$ (Fig. 4). Levels of other selenoprotein mRNAs, such as SelH and SelS, and possibly SelM, SelV, and TR2 also appeared to be down in mice carrying *trsp^t*i^{6}A$^-$, suggesting that these proteins, along with GPx2, GPx3, SelR, SelT, and SelW, may not be rescued or may be only partially rescued. DI1, GPx4, SelK, SelP, and Sep15 appeared to have similar amounts of mRNA in mice carrying *trsp* and *trsp*i^{6}A$^-$. The observation that some mRNA levels appeared to be unaffected is interesting in light of the fact that the corresponding selenoproteins, namely GPx4, SelP, and Sep15, are only partially rescued as demonstrated by ^{75}Se-labeling and/or Western blot analysis (see above). In addition, some selenoprotein mRNAs, such as SelM, SelT, and TR1, appeared to be enriched in mice carrying *trsp^t* (Fig. 4).

Glutathione Peroxidase Activities—It was surprising to find that GPx1, GPx2, and GPx3 appeared to be slightly enriched as observed by ^{75}Se-labeling and/or Western blot analysis in certain tissues in mice carrying *trsp^t* (see above), since previous studies suggested that Sec tRNA$^{[Ser]Sec}$ was not limiting in

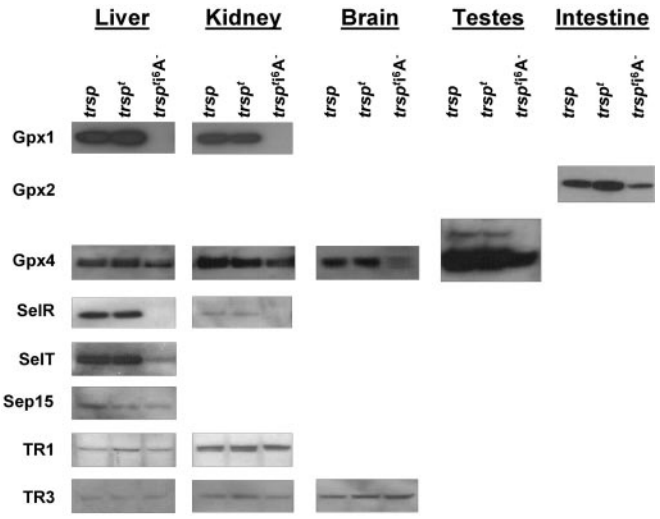

FIG. 3. **Western blot analyses.** Protein extracts were prepared from the tissues shown of mice carrying *trsp*, *trsp*^{t}, and *trsp*^{t}i^{6}A$^-$, electrophoresed, transblotted onto membranes, and treated with the appropriate antibodies as described under "Experimental Procedures." Selenoproteins are shown on the *left* of the *panels*.

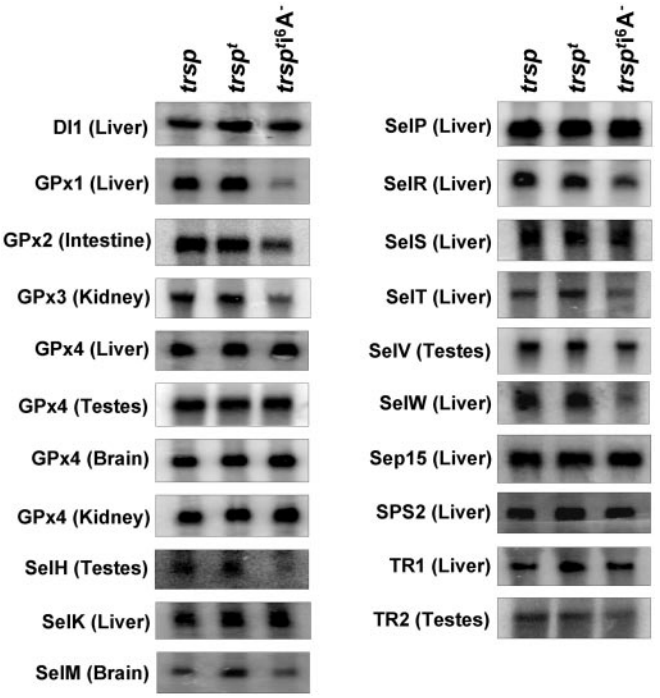

FIG. 4. **Northern blot analyses.** RNA was extracted from tissues of mice carrying *trsp*, *trsp*^{t}, and *trsp*^{t}i^{6}A$^-$, electrophoresed on gels, transblotted onto membranes, and hybridized with the appropriate probe, and the amount of labeling was determined on a PhosphorImager as given under "Experimental Procedures." Staining of developed gels with ethidium bromide showed virtually identical amounts of 18 and 28 S rRNA present in all tissue extracts (data not shown), demonstrating that the same amounts of RNA were loaded onto gels. Each of the above Northern blots was carried out at least three separate times with tissues from different mice with similar results.

selenoprotein biosynthesis (11, 29, 36). To further assess the enrichment of the glutathione peroxidases, we assayed their activity in various tissues (Table I). The assay did not distinguish between the different peroxidases but did assess whether GPx activity was increased or decreased. GPx (most likely GPx1) activity appeared to be only slightly increased in liver and heart, but not in kidney. The highest increase in GPx activity (most likely GPx2) was observed in intestine. Although

TABLE I
Glutathione peroxidase activity

GPx activity was measured as described under "Experimental Procedures." Each assay was carried out in duplicate, and the *numbers* represent the average of these two values. The two activities for each tissue represent values obtained from two different mice with the same genotype.

Tissue	GPx activity[a]		
	trsp	*trsp*^{t}	*trsp*^{t}i^{6}A$^-$
Liver	94.9	113.8	3.1
	114.0	159.6	3.6
Kidney	125.3	116.5	9.1
	116.3	135.2	3.0
Plasma	600.8	790.4	52.7
	416.0	444.9	37.5
Heart	7.5	10.7	4.8
	12.1	13.4	3.0
Intestine	9.2	15.3	0.9
	11.9	12.5	0.6

[a] GPx activities are expressed as nmol of NADPH/min/mg of protein for liver, kidney, heart, and intestine. Plasma values are expressed as nmol of NADPH/min/ml.

^{75}Se labeling of selenoproteins suggested that GPx3 might be enriched in plasma of mice carrying *trsp*^{t} (see Fig. 2), its activity was increased only slightly. Further studies are needed, however, to examine increases in GPx expression and activity due to overexpression of Sec tRNA$^{[Ser]Sec}$.

Selenium Status—Selenium levels in liver, heart, brain, and testes of mice carrying *trsp*, *trsp*^{t}, and *trsp*^{t}i^{6}A$^-$ were determined. The amounts of this element were reduced in these tissues of mice carrying *trsp*^{t}i^{6}A$^-$ as compared with the corresponding tissues for wild type mice as shown in Fig. 5. The greatest loss in selenium content, as well as relative overall loss, was in liver of mice carrying *trsp*^{t}i^{6}A$^-$. The relative loss in the brain and testes was surprisingly greater than that in the heart, since the former two tissues are known to maintain their selenium levels in virtually the same amounts even when the animal is subjected to selenium-deficient diets (37, 38). On the other hand, the levels of this element in heart and liver are responsive to selenium status and manifest reduced levels during conditions of selenium deficiency (37, 38).

Litter Sizes and Male Fertility—Matings between wild type mice and mice carrying *trsp*^{t} resulted in normal litter sizes of about eight offspring. Matings between mice carrying *trsp*^{t}i^{6}A$^-$, however, resulted in few pregnancies and in only one or two offspring when the mating was successful. Therefore, the sperm from two male mice carrying *trsp*^{t}i^{6}A$^-$ was analyzed and compared with sperm from mice carrying *trsp* and *trsp*^{t}. The sperm from wild type males and males carrying *trsp*^{t} had greater than 90% normal morphology (data not shown). A value of ~10% abnormal morphology in sperm is not unusual for the wild type mice used in this study.[3] Sperm from the *trsp*^{t}i^{6}A$^-$ mice, however, had a high percentage of cells with distorted morphology that probably accounted for their low fertility (see Table II). Matings between females rescued with the mutant transgene and males carrying one copy of *trsp* and heterozygous or homozygous for *trsp*^{t}i^{6}A$^-$ yielded smaller litter sizes with an average of four mice/litter. Analysis of sperm showed normal morphology (data not shown), suggesting that females rescued with the mutant transgene had some aberration with fertility and/or pregnancy to term. The observation regarding the reduced number of pregnancies and litter sizes was not further examined. Otherwise, the phenotypes of mice rescued with the mutant transgene appeared to be normal.

[3] R. Awasthi, personal communication.

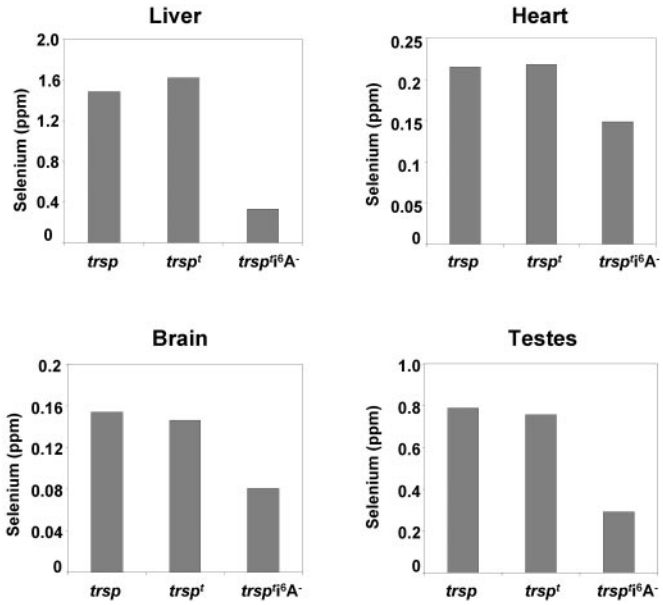

FIG. 5. **Selenium levels in tissues of mice carrying *trsp*, *trsp*^{t}, and *trsp*^{t}i^{6}A$^-$.** The amount of selenium was determined in duplicate in liver, heart, brain, and testes, and the values represent the average of assays from two separate animals of the same genotype.

TABLE II
Sperm analysis

Sperm analysis was carried as described previously (46). The sperm of two male mice of each genotype, *trsp, trsp*^{t}, *trsp*^{t}i^{6}A$^-$, and *trsp*Δ*trsp*-*trsp*^{t}i^{6}A$^{-/+}$, were examined. Sperm from all males had greater than 90% normal morphology except those of rescued mice with genotype *trsp*^{t}i^{6}A$^-$ as shown in the table.

Genotype	Age	Sperm count	Morphology		
			Normal	Bent tails[a]	Heads and tails separated
	Days		%	%	%
trsp^{t}i^{6}A-	132	45×10^6	41	56	3
trsp^{t}i^{6}A-	398	38×10^6	39	55	6

[a] Bent tails designates aberrant sperm with abnormal and bent tails with cytoplasmic droplets.

DISCUSSION

Removal of *trsp* from the mouse genome is embryonic lethal (29, 30). However, as shown in the present study, such mice can be rescued with either wild type or mutant transgenes without significantly affecting animal viability. Surprisingly, mice dependent on the mutant Sec tRNA$^{[Ser]Sec}$ transgene expressed some, but not all, selenoproteins. The mutant tRNA$^{[Ser]Sec}$ lacked i^{6}A37 and, as a consequence, was incapable of forming Um34 (8). The expression of a subset of selenoproteins is therefore dependent on one or both of these modifications. The pronounced effect of such an alteration(s) in tRNA on protein synthesis has not been previously observed.

Since the single species of tRNA$^{[Ser]Sec}$ in mice carrying *trsp*^{t}i^{6}A$^-$ lacks both i^{6}A and Um34, questions may be raised of which one of these two modifications (if not both) influence selenoprotein expression in the manner observed in this study. Clearly, the isoform lacking i^{6}A is efficiently utilized in protein synthesis, but only expressing selenoproteins such as TR1, TR3, and GPx4. The Sec tRNA$^{[Ser]Sec}$ pool is missing Um34, and the loss of this methyl group is correlated with the down-regulation of several stress-related selenoproteins such as GPx1, GPx3, SelR, SelT, and SelW. Furthermore, the addition of Um34 to tRNA$^{[Ser]Sec}_{mcmU}$ requires selenium, and selenium-deficient mice not only have reduced levels of the mcm^{5}Um isoform but also have reduced levels of several of the poorly translated selenoproteins (reviewed in Ref. 2) identified in this study. This hierarchy in selenoprotein synthesis (see Refs. 2 and 3 and this study) correlates with the presence of Um34, but not i^{6}A37. In addition, the synthesis of the methyl group on the mcm^{5}Um isoform is a highly specialized event in selenoprotein expression as noted in the Introduction. It is of significance to emphasize three earlier studies that show a direct correlation between the levels of Um34 on tRNA$^{[Ser]Sec}$ and GPx1. Replenishment of selenium-deficient rats with this element showed a direct correlation between increasing amounts of tRNA$^{[Ser]Sec}_{mcmUm}$ and GPx1 (10). Transgenic mice encoding the Sec tRNA$^{[Ser]Sec}$ i^{6}A37 minus transgene manifested a pronounced decrease in both the Um34 isoform and GPx1 (11). More recently, a study by Jameson and Diamond (12) demonstrated a preferential use of tRNA$^{[Ser]Sec}_{mcmUm}$ in selenoprotein synthesis when GPx1 was overexpressed. Therefore, the evidence supports a role of this methyl group in the synthesis of several selenoproteins that are down-regulated in the rescue mouse described in this study. Caution must be exercised in the interpretation that Um34 is solely responsible for the observed effects, since we cannot rule out a possible influence of i^{6}A37. However, the Um34 modification clearly has a dramatic influence on selenoprotein expression as evidenced by the occurrence of similar selenoprotein levels detected during selenium deficiency and in the loss of Um34 on Sec tRNA$^{[Ser]Sec}$ in the present study.

How then can the absence of Um34 play a major role in the expression of some, but not all, selenoproteins? An examination of many different parameters that might be involved in decoding efficiency of the selenoproteins identified in the present study as being poorly translated in rescued mice (GPx1, GPx3, SelR, SelT, and SelW), partially translated (GPx2, GPx4, SelP, and Sep15), and efficiently expressed (TR1 and TR3) as well as the 13 other selenoproteins in mice (13) did not reveal any specific pattern that would appear to influence translation. Other features such as the possibility of specific SECIS-binding proteins that could act specifically on different selenoproteins will have to await further investigation. The finding in the present study, however, that the presence of two Sec isoforms differing by a methyl group are utilized differently in selenoprotein biosynthesis is highly significant and demonstrates a unique manner in which Sec machinery has evolved in higher animals to express this class of proteins under conditions of selenium deprivation.

The mice that were rescued with the mutant Sec tRNA$^{[Ser]Sec}$ transgene in this study appeared to have a normal phenotype with the exception of reduced fertility in males and an apparent reduced litter size in females. The loss of the nuclear form of GPx4 and the reduced levels of GPx4 in testes probably contributed to the loss in male fertility. In addition, these results provide further evidence of a direct role of selenium in mammalian male fertility (39). Mice dependent on *trsp*^{t}i^{6}A$^-$ were also selenium-deficient in the organs tested, suggesting that most of the selenium content of tissues is contained in selenoproteins. These observations are consistent with an earlier study in which the targeted removal of selenoprotein expression in mouse liver resulted in the loss of most of the selenium in this organ, whereas the amount of small molecular weight selenocompounds did not change from that found in wild type livers (see Ref. 31 and references therein).

Mammalian selenium-containing proteins respond to changes in selenium status in a selenoprotein- and tissue-specific manner, but the mechanisms for this hierarchy (2, 3) and specific roles of many selenoproteins (13) have not been resolved. Our data suggest that the biosynthesis of GPx1, GPx3, SelR, SelT, and SelW is dependent on tRNA$^{[Ser]Sec}_{mcmUm}$, whereas that of others, such as TR1 and TR3, is not. The group

of selenoproteins that are not rescued in the $trsp^{t}i^{6}A^{-}$ mice includes stress-related selenoproteins, GPx1, GPx3, and SelR, whereas SelT and SelW also probably fall into this category. Expression of several of these proteins is known to be affected by selenium deficiency, which also results in decreased levels of their mRNAs due to nonsense-mediated decay (40, 41). The data presented herein also suggest that some selenoproteins such as GPx4, Sep15, and SelP are partially rescued, but their mRNA levels were not affected. Determination of whether selenoprotein hierarchy may be regulated by Um34 in Sec $tRNA^{[Ser]Sec}$ and whether the corresponding mRNAs may not be translated and subject to nonsense-mediated decay, and therefore whether the second event is the result of the first, must await further study.

The group of selenoproteins that were fully rescued in the $trsp^{t}i^{6}A^{-}$ mice included TR1 and TR3 that are less affected by selenium deficiency (2, 3). TR1 and TR3 control the redox state of cytosolic and mitochondrial thioredoxins, respectively, which are essential mammalian proteins.

Nucleoside modifications within the tRNA anticodon loop have been associated with a variety of effects on translation (reviewed in Ref. 42). In higher eukaryotes, such modifications have been shown to influence ribosomal frame shifting (43) and suppression of nonsense codons (44, 45). Although we have known for many years that the methyl modification at position 34 in Sec $tRNA^{[Ser]Sec}$ is a highly specialized event (see Introduction), this study provides the first example of translation of a number of mRNAs being dependent on the recoding of an in-frame nonsense codon by Um34 and describes a new and novel role of tRNA modification in the recoding process.

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