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# Rat skeletal muscle selenoprotein W: cDNA clone and mRNA modulation by dietary selenium

**(selenocysteine insertion sequence element/selenium deficiency myopathy)**

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**ABSTRACT** **Rat skeletal muscle selenoprotein W cDNA was isolated and sequenced. The isolation strategy involved design of degenerate PCR primers from reverse translation of a partial peptide sequence. A reverse transcription-coupled PCR product from rat muscle mRNA was used to screen a muscle cDNA library prepared from selenium-supplemented rats. The cDNA sequence confirmed the known protein primary sequence, including a selenocysteine residue encoded by TGA, and identified residues needed to complete the protein sequence. RNA folding algorithms predict a stem–loop structure in the 3′ untranslated region of the selenoprotein W mRNA that resembles selenocysteine insertion sequence (SECIS) elements identified in other selenocysteine coding cDNAs. Dietary regulation of selenoprotein W mRNA was examined in rat muscle. Dietary selenium at 0.1 ppm as selenite increased muscle mRNA 4-fold relative to a selenium-deficient diet. Higher dietary selenium produced no further increase in mRNA levels.**

Selenium deficiency in lambs and calves results in white muscle disease, a disorder characterized by degeneration of both skeletal and cardiac muscle (1). In humans, severe dietary selenium deprivation in discrete regions of China is associated with an endemic juvenile cardiomyopathy called Keshan disease (2). Muscle weakness in patients on long-term parenteral nutrition can be alleviated and prevented by selenium supplementation (3, 4). Therefore, selenium is important for normal muscle metabolism.

Mammalian selenoenzymes have two known functions at present. The selenium-dependent glutathione peroxidase (GSHPx) family has at least four members that protect against the deleterious effects of peroxides and hydroperoxides in both cellular and extracellular compartments of tissues. These include the cellular GSHPx glutathione: $H_2O_2$ oxidoreductase (E.C. 1.11.1.9) (5, 6); the plasma GSHPx-p (7); the phospholipid hydroperoxide glutathione peroxidase (8), which reduces fatty acid hydroperoxides esterified to phospholipids; and a fourth enzyme found primarily in the gastrointestinal tract, designated GSHPx-GI (9).

The other known mammalian selenoenzyme is type I iodothyronine 5′-deiodinase (5′DI) (10, 11). The action of 5′DI is to convert thyroxine to triiodothyronine, thus providing a link between selenium and iodine. All of these selenoenzymes contain one selenium atom per polypeptide chain although some of the GSHPxs are multimeric proteins. The genes for all these enzymes have been cloned. Dietary selenium regulates, in a tissue-specific fashion, the enzymatic activities of cellular GSHPx, phospholipid hydroperoxide glutathione peroxidase, and 5′DI and levels of their mRNAs (12–14).

Although its function remains unknown, the majority of the selenium in plasma of rats (15) and humans (16) is associated with selenoprotein P. The cDNA for selenoprotein P has been cloned and sequenced (17). There are 10 TGAs, the selenocysteine (Sec) codon, in the open reading frame, indicating that the protein contains 10 Sec residues. The levels of this protein in the plasma are affected by the selenium status of the animal (18). The only other selenoprotein known to possess >1 Sec per subunit is mitochondrial capsule selenoprotein from mouse sperm, which contains 3 Sec residues (19). Likewise, its function remains unknown. Some evidence for a number of other selenoproteins has been presented (20–22), and we have reported the properties of selenoprotein W, a low molecular weight protein from rat skeletal muscle (23).

Selenoprotein W, isolated from cytosol, is a single polypeptide containing 1 mol of selenium per mol. Purification yields a mixture of four molecular weight forms, the smallest of which is 9550 Da. These multiple forms arise from binding of reduced GSH or an unidentified 45-Da moiety or both (24). Selenoprotein W is widely distributed in tissues of rat and sheep and is present in the greatest quantities in rat skeletal muscle and both skeletal and cardiac muscle of sheep (25, 26). The level of the protein in skeletal muscle and some other tissues is regulated by dietary selenium (25, 26). Western blots of human and rhesus monkey tissues using polyclonal antibodies raised against a synthetic peptide derived from rat muscle selenoprotein W yielded a positive signal only in heart and skeletal muscle (Q. Gu, J.-Y.Y., M.A.B., N. E. Forsberg, and P.D.W., unpublished data). Although the function of selenoprotein W has not been determined, its species-specific tissue distribution, selenium dependence, and binding to GSH suggest a role in the myopathies of selenium deficiency.

In this study, we isolated and sequenced a cDNA copy of the selenoprotein W gene† and demonstrated that dietary selenium status altered the abundance of its mRNA. The secondary structure of the mRNA contained a putative Sec insertion sequence element (SECIS) similar to those described for other Sec-encoding mRNAs (27).

## MATERIALS AND METHODS

**Reverse Transcription-Coupled PCR to Produce a Selenoprotein W Probe.** One female Sprague–Dawley rat (Simonsen Laboratories, Gilroy, CA) was fed a torula yeast-based diet (23) containing selenium at 1 ppm as sodium selenite for 10 days. The animal was anesthetized with sodium pentobarbital, bled by cardiac puncture, and killed by pneumothorax. Skeletal muscle was removed from the hindlegs and immediately frozen in liquid nitrogen.

Total RNA was isolated from 9.6 g of tissue by the single-step guanidinium isothiocyanate method (28). The final pelleted RNA was dissolved in diethylpyrocarbonate-treated water to 1.65 mg/ml by heating for 10 min at 65°C. Poly(A)$^+$ RNA

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Abbreviations: GSH, glutathione; GSHPx, GSH peroxidase; Sec, selenocysteine; SECIS, Sec insertion sequence; 5′DI, 5′-deiodinase.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U25264).

was prepared from 7.8 mg of total RNA by passage through an oligo(dT)-cellulose column twice by standard procedures (28), further washed with 70% ethanol, and reprecipitated to remove traces of LiCl and SDS prior to cDNA synthesis. Approximately 100 μg of total RNA enriched in poly(A)$^+$ RNA was reverse-transcribed for 60 min at 42°C with oligo(dT) or random primers to generate first-strand cDNA by using the cDNA cycle kit from Invitrogen. The reverse transcription reaction was repeated after denaturation at 95°C for 2 min by addition of fresh enzyme. The PCR was carried out on the cDNA·RNA hybrid using two degenerate oligonucleotide primers based upon the peptide sequence of selenoprotein W (29). The sense and antisense strand primers were 29 and 26 bp long, respectively. The forward primer was derived from the peptide sequence from amino acids 23–32 (KEKLEHEFPG) (5′-AAA/GGAA/GAAA/GCTIGAA/GCAT/CGAA/GTTT/CCCIGG-3′) and the reverse primer was from the peptide sequence from amino acid residues 50–42 (VEFFGTQP) (5′-ACC/TTCA/GAAA/GAAICCIGTIACC/TTGIGG-3′). The PCR was performed in a total volume of 10 μl in glass capillary tubes with final concentrations of 58 mM KCl, 30 mM Tris·HCl (pH 8.3), 4.5 mM $MgCl_2$, all four dNTPs (each at 0.25 mM), 0.001% gelatin, bovine serum albumin (0.5 ng/ml), each oligonucleotide primer at 1.5 μM, and 0.4 unit of *Thermus aquaticus* (*Taq*) DNA polymerase (Promega) by rapid cycle DNA amplification (Idaho Technology, Idaho Falls). Samples were denatured at 95°C for 2 sec followed by annealing at 48°C for 2 sec and 15 sec of extension at 72°C for 50 cycles. The resulting 83-bp PCR product (corresponding to bases 117–199, Fig. 1*A*) was reamplified, gel-purified, cloned (TA cloning kit, Invitrogen), and sequenced. DNA primers and probes were synthesized by using Applied Biosystems model 380 automated DNA synthesizer.

**cDNA Library Construction.** Double-stranded cDNA was synthesized from 5 μg of poly(A)$^+$-enriched RNA by reverse transcription with oligo(dT) primers. The cDNA was packaged into λ ExCell by using kits from Pharmacia/LKB (cDNA synthesis kit and Ready-To-Go λ packaging kit) to obtain $7 \times 10^5$ recombinant phages at an efficiency of $1 \times 10^6$ plaque-forming units/μg of DNA. Approximately $1.5 \times 10^5$ plaques were screened to obtain 9 positive clones. The cDNA clones were converted to pExCell plasmids by *in vivo* phagemid release.

Three cDNA clones were sequenced from the vector encoded SP6 and T7 promoters by using the dye-primer cycle protocol with Applied Biosystems model 373A automated DNA sequencer. Further sequencing from the interior of the cDNA sequence was done by DyeDeoxy chain termination method (31) using the sense primer 5′-TCAAAGACCTGTGGTCTTTCTTCGATG-3′ and the antisense primer 5′-ACCTGGGGAGTCCCCTCGCCACAGATG-3′, corresponding to nt 397–423 and nt 156–182, respectively (Fig. 1*A*).

**Dietary Selenium and mRNA Levels.** Two experiments were conducted to examine the relationship of dietary selenium to skeletal muscle selenoprotein W mRNA levels. Male weanling rats were fed purified diets containing selenium added as sodium selenite to 0, 0.3, 1.0, and 4.0 ppm for 4 weeks (one rat at each dietary level). In a second experiment, groups of male weanling rats were fed the same purified diets containing supplemental selenium at 0, 0.1, and 4.0 ppm for 6 weeks. The diets were shown by analysis to contain selenium at the following levels: basal, 0.002 ppm; selenite-supplemented, 0.10, 0.30, 0.97, and 3.96 ppm. Skeletal muscle was rapidly excised from the hindlegs and immediately frozen in liquid nitrogen. Total RNA was prepared from 100 to 300 mg of tissue. RNA was denatured in 20 mM Mops/48% (vol/vol) formamide/6.7% (vol/vol) formaldehyde at 100°C for 2 min and electrophoresed on 2.5% agarose in 20 mM Mops, pH 6.0/2% formaldehyde. RNA was transferred onto nylon membrane (GeneScreen*Plus*, DuPont/NEN) and hybridized with a

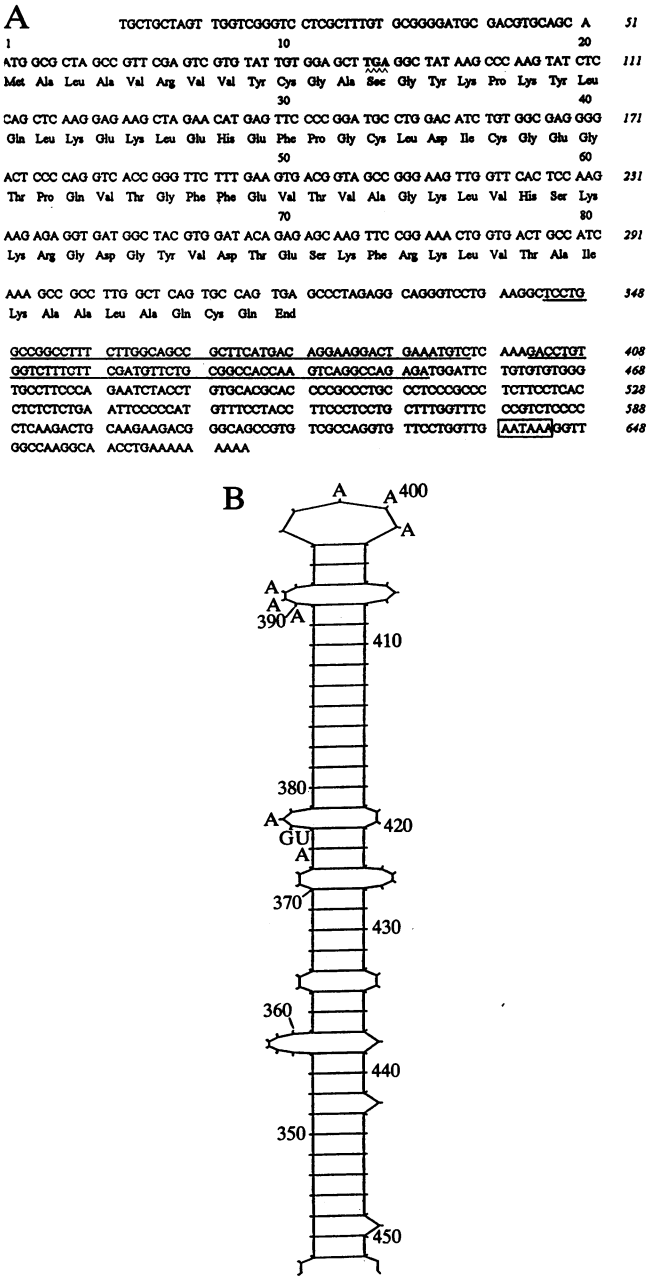


FIG. 1. (*A*) cDNA and amino acid sequences of rat selenoprotein W. The TGA (residues 88–90) encoding Sec (amino acid residue 13) is shown in boldface type (~~). The arms of a putative SECIS element (residues 344–396 and 402–451) are underlined. A possible polyadenylylation signal (residues 639–644) is boxed. Numbers in italic type refer to nucleotide sequence. (*B*) Structure of the putative SECIS element (bases 344–451) in 3′ untranslated region of selenoprotein W mRNA. Predicted structure derived by using RNAFOLD program (30). Labeled 5′ sequences correspond to the consensus SECIS structure (27).

labeled oligonucleotide probe (inverse complement of bases 156–182, Fig. 1*A*) at 42°C in 50% formamide/10% (wt/vol) dextran sulfate/1.0 M NaCl/1% SDS/salmon sperm DNA (0.1 mg/ml). The membrane was washed sequentially in 2× SSC, 2× SSC/1% SDS, and 0.1× SSC/1% SDS at 42°C. Determination of the hybridization signal was done by phosphor screen autoradiography (PhosphorImager SI model PSI-486) and the signal was quantified by using IMAGEQUANT (Molecular Dynamics). In addition to the selenoprotein W mRNA probe, a 27-base oligonucleotide probe for glyceraldehyde-3-phosphate dehydrogenase (Clontech) was also hybridized to the total

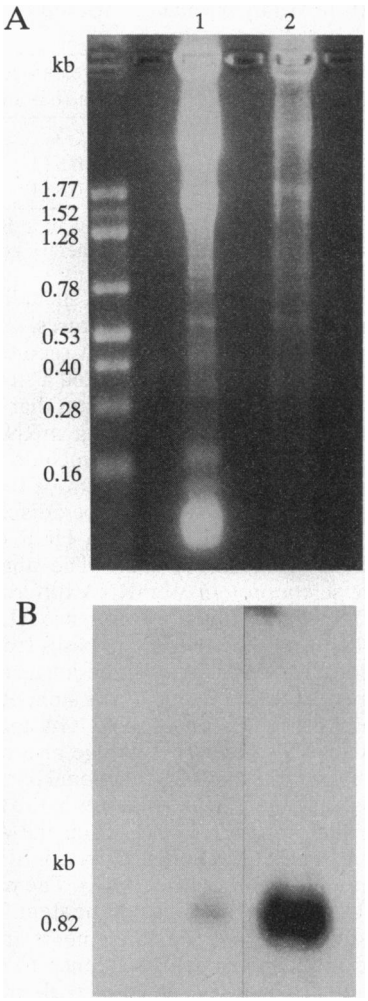


FIG. 2. Northern blot analysis of RNA from rat skeletal muscle. (*A*) Ethidium bromide-stained 2.5% agarose gel with 2.0% formaldehyde containing samples of total RNA (10 μg) and poly(A)$^+$ RNA (4 μg) in lanes 1 and 2. (*B*) Hybridization to the selenoprotein W-specific probe. RNA markers in kilobases were to estimate size.

RNA samples to detect differences in the amounts of total RNA loaded onto gels.

## RESULTS

**Selenoprotein W cDNA Sequence.** To develop perfect-match hybridization probes for the selenoprotein W gene, we used degenerate oligonucleotide primers to amplify and sequence a central part of the cDNA sequence. Primers for PCR were designed by reverse translation of segments of the peptide sequence (29) where there were no amino acid uncertainties. Primer design incorporated rat-specific codon usage frequencies, the probability for certain dinucleotide sequences, and deoxyinosine substitution at sites of 4-fold codon degeneracy. First-strand cDNA synthesis was achieved with both random and oligo(dT) priming of skeletal muscle poly(A)$^+$ RNA. Amplification of cDNA·RNA hybrids resulted in an 83-bp product. The PCR product yielded 38 bp of nucleotide sequence that lies between the primers. Based on this information, several 27-base oligonucleotide sequences were synthesized and used to probe the cDNA library and Northern blots.

Three cDNA clones had identical nucleotide sequences except for slight differences in the lengths of the 5′ untranslated sequences and the poly(A) tail. The cDNA sequence in Fig. 1*A* is composed of 672 nt. The 5′ untranslated region consists of 51 bases followed by the 267-base coding sequence. The coding region includes the Met start codon, ATG, and a termination codon, TGA. The 3′ untranslated region is composed of 345 bases with an additional 9 bases of poly(A) present at the terminus. The presence of the Sec codon, TGA, in the position that corresponds to amino acid residue 13 in the predicted sequence confirms that the protein is indeed a selenoprotein. The nucleotide sequence has confirmed the published amino acid sequence (29) and revealed the identity of previously unknown residues. The following amino acid residues were identified: Cys-10, -33, and -37, Ser-71, Lys-72, and positions 78–88 as Thr-Ala-Ile-Lys-Ala-Ala-Leu-Ala-Gln-Cys-Gln.

Selenoprotein W mRNA 3′ untranslated sequence was analyzed by using the Genetics Computer Group (Madison, WI) FOLDRNA program (30) to detect secondary structures that are similar to the SECIS elements reported for other Sec-encoding mRNAs (27). The predicted stem–loop structure is shown in Fig. 1*B*.

**Selenium-Dependent Accumulation of Selenoprotein W mRNA.** Northern blot analyses of rat skeletal muscle total RNA and poly(A)$^+$ RNA are shown in Fig. 2. The message size was estimated at 820 bases. Total RNA from skeletal muscle

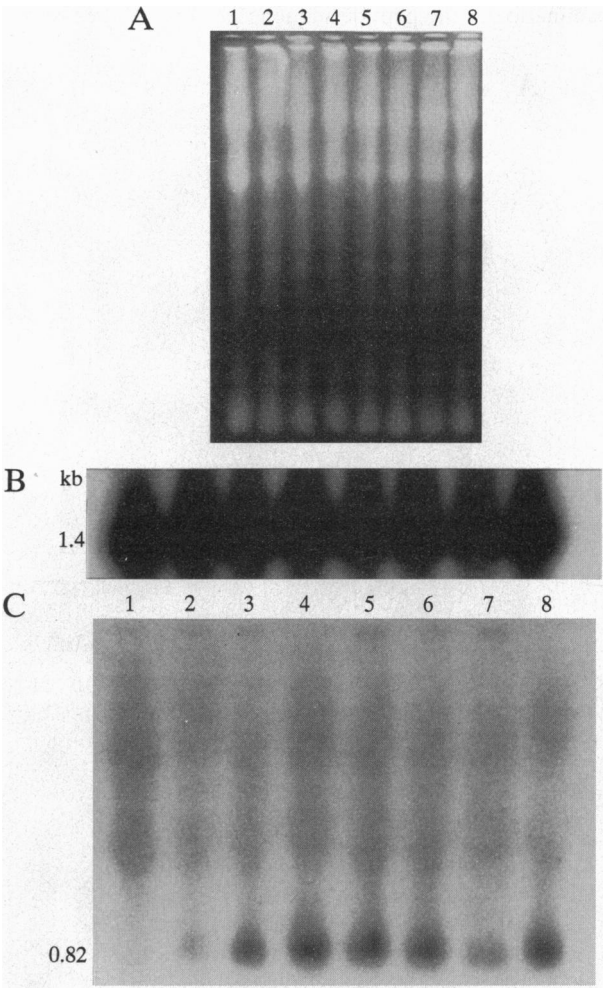


FIG. 3. Northern blot analysis of skeletal muscle RNA from rats fed various levels of selenium (experiment 1). Duplicate 50-μg samples of RNA were electrophoresed on 2.5% agarose gel containing 2.0% formaldehyde (*A*). Hybridizations to glyceraldehyde-3-phosphate dehydrogenase (*B*) and selenoprotein W-specific probes (*C*) were done for animals given selenium at 0 ppm (lanes 1 and 2), 0.3 ppm (lanes 3 and 4), 1.0 ppm (lanes 5 and 6), and 4.0 ppm (lanes 7 and 8). These data support no further change in muscle selenoprotein W mRNA content for dietary selenium intakes between 0.1 ppm and 4.0 ppm as shown in Table 1.

of rats receiving different levels of dietary selenium was subjected to Northern blot analysis as shown in Figs. 3 and 4. The ratios of mRNA hybridization of probes for selenoprotein W and glyceraldehyde-3-phosphate dehydrogenase for each sample were determined. The mRNA levels in animals fed supplemented diets are expressed as fold increases over the levels measured in rats fed the basal diet (Table 1). Selenium supplementation at 0.1 ppm increased selenoprotein W mRNA to levels 4-fold higher than those achieved by rats subjected to selenium deficiency. Statistically significant differences in hybridization ratios were obtained between the basal diet (no added selenium) and the supplemented diets ($P < 0.001$) but there was no significant difference between the two levels of supplementation (Table 1).

## DISCUSSION

Cloning the cDNA for selenoprotein W led to the completion, confirmation, and correction of the previously reported amino acid sequence (29). In our previous work (23) Trp was mistakenly indicated in the selenoprotein W amino acid composition. This should have been Tyr, as noted later (25). Examination of the peptide sequence around the Sec residues

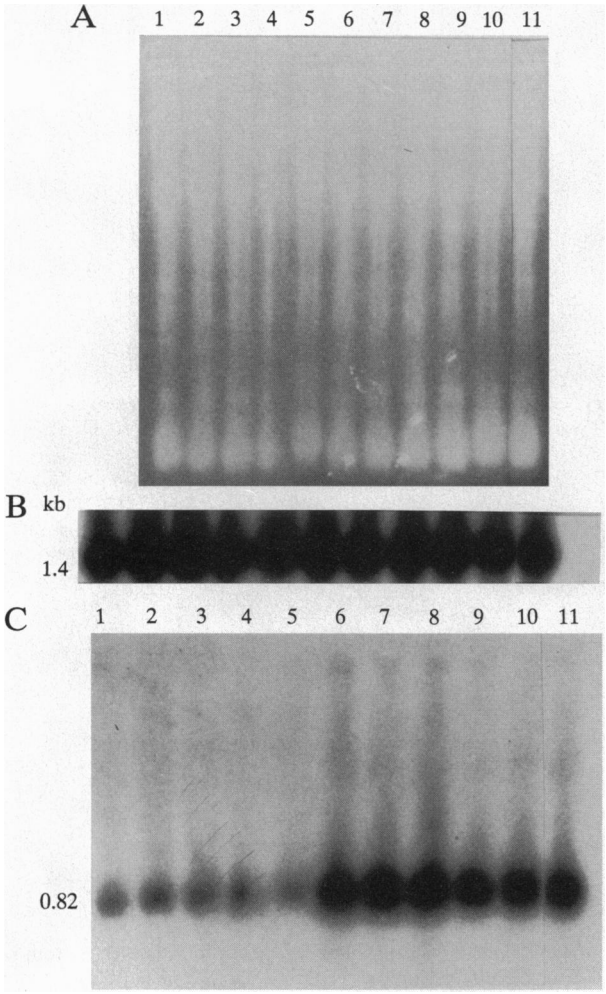


FIG. 4. Northern blot analysis of skeletal muscle RNA from rats fed various levels of selenium (experiment 2). Fifty-microgram samples of RNA were on 2.5% agarose gel containing 2.0% formaldehyde (*A*). Hybridizations to glyceraldehyde-3-phosphate dehydrogenase (*B*) and selenoprotein W-specific probes (*C*) were done for animals given selenium at 0 ppm (lanes 1–5), 0.1 ppm (lanes 6–9), and 4.0 ppm (lanes 10 and 11).

Table 1. Effects of dietary selenium on selenoprotein W mRNA levels

| Added dietary selenium, ppm | Fold increase over basal diet (0 ppm) |
|---|---|
| 0 | 1 (6) |
| 0.1 | 4.0 ± 0.5 (4) |
| 4.0 | 4.4 ± 0.5 (3) |

Values for supplemented groups are the mean ± SD. Numbers in parentheses denote number of rats in each dietary group.

of 10 selenoproteins (11, 17, 19, 32–36), including selenoprotein W (29), revealed no conserved features.

Prediction of selenoprotein W mRNA secondary structure using the FOLDRNA program (30) indicated a stem–loop structure (Fig. 1*B*) in the 3′ untranslated region that resembles the SECIS elements of other Sec-encoding mRNAs (27). The conserved features of these structures include (*i*) a partially unpaired sequence AUGA in the 5′ arm of the stem 10–12 bases below the loop that contains three adjacent adenosine residues and (*ii*) an unpaired sequence, UGR (where R is a purine), in the 3′ arm of the stem. The apparent SECIS element in the selenoprotein W mRNA differs slightly from this pattern. The partially unpaired sequence AUGA occurs in the 5′ side of the stem separated by 19 bases from the start of the terminal loop, which includes three adjacent adenosines. An unpaired region on the 5′ side of the stem occurs between the "bulge" containing the sequence AUGA and the terminal loop of the stem. This intervening bulge also contains three adjacent adenosines that may be a functional part of the SECIS element, rather than the three adenosines in the terminal loop. The triplet in the bulge is 12 bases from the AUGA bulge, which makes the separation similar to that for SECIS elements identified in other selenoprotein mRNAs. The partially paired sequence UGU occurs in the 3′ side of the stem. The structural requirements for functional SECIS elements are not entirely known, so the availability of this sequence to compare with those identified previously may help to define the critical features.

The UGA codon signals both Sec insertion and termination in selenoprotein W mRNA (Fig. 1*B*). Failure to interpret the termination codon as a Sec residue probably results from its proximity to the SECIS element. Limits on the proximity of SECIS elements to UGA codons encoding Sec are not known. All mammalian selenoproteins except selenoprotein P have their Sec residues close to the N terminus. Although 9 out of 10 Sec residues in selenoprotein P occur relatively close to the C terminus, a 3′ untranslated region ≈240 bases long separates the final Sec from the start of the stem of the SECIS element (17). The stem of the putative SECIS element in selenoprotein W mRNA begins only 26 bases from the termination UGA, but it is 255 bases from the UGA encoding Sec. Proximate contextual signals are apparently not involved in UGA interpretation. Berry *et al.* (27) found that out-of-context UGA codons introduced at five positions in 5′DI message were suppressed by functional SECIS elements in the 3′ untranslated region. The GSHPx mRNA of *Schistosoma mansoni* (37) also uses an UGA for both Sec insertion and termination. The mechanism for discrimination of the functions of the UGA codons may be similar for both selenoprotein W and *Schistosoma* GSHPx mRNAs.

The increase of rat skeletal muscle SelW mRNA with selenium exposure is consistent with previous work demonstrating Se modulation of muscle-tissue selenoprotein W levels (25). Although selenoprotein W mRNA accumulation is altered by dietary selenium status, the levels of message and protein do not respond in parallel. Although there was no change in selenoprotein W mRNA level with dietary selenium between 0.1 and 4.0 ppm (Fig. 4 and Table 1), there was an 8-fold increase in protein content (25). This suggests that

selenium influences the concentration of selenoprotein W by a mechanism that is not limited to its effect on the level of selenoprotein W message and may involve translation.

The function of selenoprotein W is unknown but, like selenium-dependent GSHPx (38), it can be isolated from muscle tissue with bound GSH (24). This suggests a possible role in oxidation/reduction catalysis. Selenium deficiency disorders such as human Keshan disease and white muscle disease of sheep may be related to the function of this protein. Oxidative damage may cause the pathological changes in cardiac muscle in Keshan disease (39). Ultrastructural and biochemical aberrations in mitochondria precede the necrotic deterioration of cardiac muscle characteristic of human Keshan disease (40). Evidence for enhanced lipid peroxidation in skeletal muscle mitochondria in selenium-deficient exercising rats has been reported (41). Sufficient evidence exists to warrant further investigation into a possible link between selenoprotein W and selenium deficiency myopathies. The highest concentrations of selenoprotein W are found in skeletal and cardiac muscle tissue of susceptible species (unpublished data and ref. 26). Substantial reductions in both the protein and its mRNA occur in these tissues in response to dietary selenium deprivation. Characterization of selenium-dependent transcriptional and post-transcriptional regulation of selenoprotein W mRNA levels, in various species and tissues, should contribute to understanding the biochemical and physiologic function of this protein and its relationship to the heart and skeletal muscle myopathies of selenium deficiency.

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