Mammalian thioredoxin reductases contain a TGA-encoded C-terminal penultimate selenocysteine (Sec) residue, and show little homology to bacterial, yeast, and plant thioredoxin reductases. Here we show that the nematode, Caenorhabditis elegans, contains two homologs related to the mammalian thioredoxin reductase family. The gene for one of these homologs contains a cysteine codon in place of TGA, and its product, designated TR-S, was previously suggested to function as thioredoxin reductase. The other gene contains TGA and its product is designated TR-Se. This Sec-containing thioredoxin reductase lacks a canonical Sec insertion sequence element in the 3'-untranslated area of the gene. TR-Se shows greater sequence similarity to mammalian thioredoxin reductase isozymes TR1 and TR2, whereas TR-S is more similar to TR3. TR-Se was identified as a thioredoxin reductase selenoprotein by labeling C. elegans with 75Se and characterizing the resulting 75Se-labeled protein by affinity and other column chromatography and gel-electrophoresis. TR-Se was expressed in Escherichia coli as a selenoprotein when a bacterial SECIS element was introduced downstream of the Sec TGA codon. The data show that TR-Se is the major naturally occurring selenoprotein in C. elegans, and suggest an important role for selenium and the thioredoxin system in this organism.

Mammalian thioredoxin reductases (1) are members of the Type I pyridine nucleotide–disulfide oxidoreductase enzyme family, which also includes several other enzymes such as glutathione reductase and lipoamide dehydrogenase. The members of this family are homodimers of 50-65 kDa subunits and each subunit contains a flavin adenine dinucleotide cofactor. These enzymes utilize NADPH to reduce natural substrates and the reduction takes place at the disulfide active center located in the N-terminal portion of each enzyme (2). Mammalian thioredoxin reductases are different from bacterial, plant and yeast thioredoxin reductases, which are homodimers of ~35 kDa subunits, have different reaction mechanisms, and have little homology to Type I pyridine nucleotide–disulfide oxidoreductase enzymes.

The difference between mammalian thioredoxin reductases and other enzymes of the Type I pyridine nucleotide–disulfide oxidoreductase family is that thioredoxin reductases contain a C-terminal extension with a conserved C-terminal Gly-Cys-Sec-Gly (Sec is selenocysteine) tetrapeptide (3,4). The Sec residue in thioredoxin reductases has been implicated in the enzymes' catalytic activity (5-7), in their broad substrate specificity (8-12) and in their inhibition by gold and alkylating compounds (13-16). Furthermore, Sec in mammalian TR1 has been suggested to be involved in redox regulation of cell signaling (17).

In addition to thioredoxin reductases, Sec, encoded by a TGA codon, occurs in eleven other, known mammalian proteins. However, homologous sequences from invertebrates and lower eukaryotes generally contain a cysteine (Cys) codon in place of TGA, implying that Sec is very rare or absent in these organisms. In the course of initial computer sequence analyses of animal thioredoxin reductases, we (18) and others (19) observed a high homology between mammalian thioredoxin reductase sequences and two gene products in Caenorhabditis elegans. Interestingly, one of the putative C. elegans thioredoxin reductase genes contained a
conserved Sec-encoding TGA codon. A Sec tRNA gene has been previously found in this organism (20) suggesting that C. elegans has the ability to synthesize selenoproteins. In this report, we present the complete cDNA sequence for the Sec-containing thioredoxin reductase, TR-Se, and provide evidence for the natural occurrence of this enzyme in C. elegans.

MATERIALS AND METHODS

Nematode culture. C. elegans was cultured as described by Brenner (21) using Escherichia coli strain OP50. To label C. elegans with 75Se, worms were grown in liquid culture (22) in the presence of 100 μCi [75Se]selenite (1000 Ci/mmol; University of Missouri Research Reactor) for 2 days, washed with phosphate-buffered saline, cleaned by sucrose floatation (22), and disrupted in 4 volumes of 25 mM Tris-HCl, pH 7.5, 1 mM PMSE, 1 mM EDTA, 1 mM DTT. The resulting crude extract was centrifuged at 15,000 rpm for 30 min and the supernatant and pellet fractions were analyzed on SDS-PAGE gels (Novex, CA), followed by PhosphorImager analysis of radioactivity on the gels. As a control, E. coli OP50 was incubated in liquid culture conditions in the presence of 75Se in parallel with the worm sample.

Affinity isolation of TR-Se. For thioredoxin reductase isolation, the supernatant fraction of the 75Se-labeled crude extract was loaded onto a 2 ml 2'-5'-ADP-Sepharose 4B (Pharmacia) column, which was pre-equilibrated with 50 mM TRIS-HCl, pH 7.5 (buffer A). The column containing bound protein was washed with buffer A, and further with 150 mM NaCl in buffer A, followed by thioredoxin reductase elution with 1 M NaCl in buffer A. The fractions were analyzed by SDS-PAGE gels followed by the PhosphorImager analysis. 75Se-labeled mouse testis and muscle samples were obtained as described (23).

Sequencing of the TR-Se cDNA. The Lambda ZAP II clone containing the cDNA of the C. elegans TR-Se gene was found by the BLAST computer search analysis of C. elegans EST sequences. This clone was kindly provided by Dr. Y. Kohara. Lambda DNA was extracted from the phage and purified using a QIAGEN Lambda Kit. The TR-Se cDNA was sequenced using a dye terminator DNA Sequencing Kit (Perkin Elmer).

Expression of TR-Se in E. coli. The plasmid pTRc was obtained by inserting the TR-Se coding sequence into pET-21b (+) vector (Novagen) via NdeI and XhoI sites. DNA was amplified with primers: 5'-CCAGGCACATGAACTCTCCAGGATTTAC-3' and 5'-GTTGGATCGAGCTAGGGATTGGTGCCAGACCGATGC-CTAGCCTAGCCAGAAGCTGG-3'. This procedure introduced a bacterial Sec insertion sequence (SECIS) element immediately downstream of the Sec TGA codon. The introduced SECIS element was designed on the basis of the SECIS element present in the E. coli formate dehydrogenase H gene (fdhF gene) (24). The BL21(DE3) E. coli strain was used as a host. The cells were grown in LB media with 100 μg/ml ampicillin at 37°C to OD600 = 0.5 and then induced with 1mM IPTG. For labeling with 75Se, 1 nmol/ml radioactive Na2SeO3 (−8 μCi/nmol) was added at the time of induction. Growth was continued for 3 h at 37°C after induction. The cells were harvested by centrifugation at 5000 rpm for 15 min at 4°C and stored at −70°C before use. The major form of TR-Se that was expressed in E. coli by this procedure occurred as inclusion bodies, which precluded further characterization of the recombinant selenoprotein.

Computer analysis of SECIS elements. A computer program, SECISearch (25), was used to search for SECIS elements in the C. elegans genome. The program searches for SECIS elements by initially analyzing the consensus sequence, followed by analyses of a secondary structure and the energy of SECIS. The criteria used in the searches were sufficient to identify SECIS elements in all known human selenoprotein genes except the gene for Selenoprotein P. Selenoprotein P contains two SECIS elements that are characterized by a lower energy and the use of the algorithm with the energy threshold that detects Selenoprotein P SECIS elements resulted in a large (~1000) number of hits that were difficult to test manually.

RESULTS AND DISCUSSION

Computer search analysis of non-redundant and expressed sequence tag nucleotide databases with the gene for human thioredoxin reductase (TR1) revealed two C. elegans nucleotide sequences with homology to the human enzyme (Fig. 1). One C. elegans thioredoxin reductase, TR-S (accession number Z11115), was initially identified as a probable glutathione reductase based on the sequence homology, but was later suggested to be a thioredoxin reductase (3). TR-S sequence is 503 amino acid residues long (~55 kDa).

We have determined the full-size 2541-nucleotide long cDNA sequence for the second C. elegans thioredoxin reductase, TR-Se. The genomic sequence for TR-Se (accession numbers U61958 and U61947) can be derived from the recently determined sequence of the C. elegans genome. The TR-Se gene is 4.6 kb long and is composed of 7 exons.

We did not verify experimentally the translation initiation site of TR-Se sequence, but the proposed initiation site is consistent with the mobility of the recombinant TR-Se on SDS PAGE gels (see below). The full-size TR-Se sequence (Fig. 1) is composed of 667 amino acid residues and has a calculated molecular mass of a protein subunit of ~74 kDa. The two C. elegans thioredoxin reductase protein sequences, TR-Se and TR-S, exhibit 42.9% identity in a 508 residue overlap.

The C-terminal tetrapeptide that was found in mammalian thioredoxin reductases is conserved in TR-S and TR-Se. However, in contrast to mammalian thioredoxin reductases, TR-S has Cys in place of Sec. This is not surprising, since lower eukaryotes generally contain Cys homologs of mammalian Sec-containing proteins. The unexpected finding was that the C. elegans TR-Se gene contained an in-frame TGA that encodes Sec. No Sec-containing proteins have previously been described in this organism. However, a Sec tRNA gene has been found in C. elegans (20) providing strong evidence that this organism may synthesize selenoproteins.

Comparison of the C-terminal sequences in known animal thioredoxin reductases is shown in Fig. 2. A Sec/Cys (or Cys/Cys) pair is absolutely conserved in these enzymes, except in the Plasmodium falciparum and Cryptosporidium parvum thioredoxin reductases where these residues are separated by one lysine and three glycine residues.

To directly test if C. elegans contains selenoproteins and, in particular, TR-Se, this organism was metabolically labeled with 75Se. The supernatant and pellet fractions of the crude extract were analyzed by SDS
PAGE analysis followed by PhosphorImager detection of radioactivity on the resulting gels. Since C. elegans was grown on a diet containing E. coli during the period of $^{75}$Se-labeling, a control sample was included which contained only $^{75}$Se-labeled E. coli. PhosphorImager analysis of supernatant (Fig. 3, lanes 1 and 4) and pellet (lane 3) fractions revealed three prominent $^{75}$Se-labeled bands of 110, 70 and 20 kDa (some preparations also contained a faint band of 90 kDa). However, the control E. coli sample also contained the 20 and 110 kDa bands (lane 2). These $^{75}$Se-labeled species likely correspond to bacterial Sec-containing formate dehydrogenase O (FDH O, 110 kDa) and selenium-modified tRNAs (20 kDa) (26,27). Hence, only one selenoprotein band specific for C. elegans was detected.

**Fig. 1.** Multiple sequence alignment of human and C. elegans thioredoxin reductases. The following sequences have been used: hTR1, human thioredoxin reductase 1 (Accession No. S79851); hTR2, human thioredoxin reductase 2 (17); hTR3, human thioredoxin reductase 3 (17); C.e.TR-Se, C. elegans Sec-containing thioredoxin reductase; and C.e.TR-Cys, C. elegans Cys-containing thioredoxin reductase (Accession No. Z11115). The proposed translation site of TR-Se is consistent with the mobility of TR-Se on SDS-PAGE gels. The actual translation start site has not been experimentally verified. Residues identical in all five sequences are highlighted. U represents Sec. Alignment was generated with the ClustalW 1.7 program.
FIG. 2. Alignment of the C-terminal sequences of the animal-type thioredoxin reductases. Accession numbers are indicated on the right side of the figure. The following sequences have been used: CeTR-Se, *Caenorhabditis elegans* Sec-containing thioredoxin reductase; CeTR-S, *C. elegans* Cys-containing thioredoxin reductase; HuTR1, human thioredoxin reductase 1; HuTR2, human thioredoxin reductase 2; HuTR3, human thioredoxin reductase 3; PfTR, Plasmodium falciparum thioredoxin reductase; CpTR, Cryptosporidium parvum thioredoxin reductase; DmTR, *Drosophila melanogaster* thioredoxin reductase; and MdTR, *Musca domestica* thioredoxin reductase. Residues identical in all sequences are highlighted in light gray. Sec residues (and the corresponding Cys residues) are highlighted in dark gray. U represents Sec.

To test whether *C. elegans* 75Se-labeled protein band migrates similarly to the protein encoded by *C. elegans* TR-Se gene, we expressed TR-Se in *E. coli*. A SECIS element was introduced immediately downstream of the Sec TGA codon on the basis of the naturally occurring SECIS element found in *E. coli* formate dehydrogenase H. Metabolic labeling of *E. coli* that expressed TR-Se resulted in 75Se incorporation into the protein (Fig. 3, lane 6). This method has not been previously applied to express mammalian selenoproteins in bacteria because the natural systems for Sec incorporation are different between mammals and bacteria. Generation of a bacterial SECIS element immediately downstream of the Sec TGA codon in other mammalian selenoprotein genes would result in altering amino acid sequence of the protein. However, Sec in TR-Se is the C-terminal penultimate residue, and the presence of bacterial SECIS element downstream of TGA does not alter the amino acid sequence of this protein.

TR-Se, which was metabolically labeled with 75Se in *E. coli*, migrated almost identically with the 75Se-labeled protein in crude extracts of *C. elegans* suggesting that the labeled *C. elegans* protein is thioredoxin reductase. To provide further evidence that the 75Se-labeled band in *C. elegans* is indeed TR-Se, we tested the binding of this protein to an ADP-Sepharose column. This column matrix is highly specific for thioredoxin reductases and allows remarkable enrichment of the enzymes from crude extracts. We observed that the 75Se-labeled *C. elegans* protein binds to the ADP-Sepharose column with an affinity comparable of that of mammalian thioredoxin reductases (Fig. 4). The other 75Se-labeled species detected in the supernatant fraction (Fig. 4, lane 1) of the *C. elegans* crude extract were found in an ADP-Sepharose flow through fraction (lane 2). Based on the ratio of 75Se cpm to protein concentration in fractions before and after ADP-Sepharose, TR-Se was enriched over 100-fold. The level of 75Se-labeled TR-Se relative to the levels of other 75Se-containing species is different in experiments shown in Fig. 3 and Fig. 4. Variability in the amount of contaminating bacteria and/or the general age or health of the worms could contribute to these differ-

FIG. 3. Metabolic 75Se-labeling of *C. elegans* and *E. coli* expressing TR-Se. Samples, prepared as described in Materials and Methods, were separated by the 4–12% gradient SDS-PAGE gel, followed by the PhosphorImager detection of 75Se-containing proteins and tRNAs. Lanes 1 and 4 show the supernatant of the *C. elegans* crude extract; lane 2, *E. coli* OP50 crude extract; lane 3, the pellet of the *C. elegans* crude extract; lane 5, *E. coli* BL21 crude extract; and lane 6, *E. coli* BL21 crude extract expressing TR-Se. The low abundance formate dehydrogenase band is not seen in lane 6. The location of the 110 kDa selenoprotein (*E. coli* formate dehydrogenase O), 70 kDa selenoprotein (TR-Se), and 20 kDa species (*E. coli* selenium-modified tRNAs) are indicated.

FIG. 4. Enrichment of TR-Se on ADP-Sepharose column. Supernatant of the 75Se-labeled, crude extract from *C. elegans* was fractionated on an ADP-Sepharose column as described in Materials and Methods and aliquots of fractionated and unfraccionated samples were separated on a SDS-PAGE gel and 75Se-labeled bands detected as described in the legend to figure 3. Lane 1 shows the supernatant fraction of *C. elegans* crude extract prior to loading onto the ADP-Sepharose column; lane 2, the ADP-Sepharose flow through fraction; lane 3, buffer A wash; lane 4, 150 mM NaCl in buffer A wash; lane 5, 1 M NaCl in buffer A wash; lane 6, 75Se-labeled mouse testis; and lane 7, 75Se-labeled mouse muscle. The locations of *C. elegans* TR-Se (lanes 1 and 5), mouse TR2 (lane 6), mouse TR1 (lanes 6 and 7), and mouse glutathione peroxidase 1, GPX1, (lanes 6 and 7) are indicated.
ences. Figure 4 also shows the PhosphorImager analysis of $^{75}$Se-labeled proteins in mouse testis (lane 6) and muscle (lane 7). Thioredoxin reductase 2 (TR2), which is a homodimer of 65 kDa subunits and is most highly expressed in testis, migrates on a SDS PAGE gel slightly faster than the ~70 kDa TR-Se (17).

The ADP-Sepharose enriched TR-Se, as well as TR-Se that was overexpressed in E. coli, did not react with either anti-TR1, anti-TR2 or anti-TR3 antibodies (data not shown), which are antibodies against human thioredoxin reductases. Antibodies to mammalian selenoenzymes were previously designed to specifically recognize the C-terminal parts of the proteins (17).

We further attempted the direct isolation of ADP-Sepharose enriched TR-Se using DEAE-Sepharose and phenyl-Sepharose columns. TR-Se was followed during isolation by its $^{75}$Se radioactivity. TR-Se was eluted from DEAE and phenyl columns at conditions close to the TR1 elution conditions (4). However, the extremely low level of TR-Se in C. elegans precluded purification of the enzyme to homogeneity.

The presence of thioredoxin reductases in C. elegans suggests the presence of a functional thioredoxin system or systems. Such a system would normally consist of thioredoxin reductase, thioredoxin and thioredoxin peroxidase. Two types of thioredoxin peroxidases (peroxiredoxins) have been detected previously in nematodes (28). Whether the presence of two thioredoxin reductases and two peroxiredoxins is indicative of the presence of two thioredoxin systems is not known and will require further study.

The presence of two thioredoxin reductases in C. elegans is also reminiscent of the presence of multiple thioredoxin reductases in mammalian cells. Three thioredoxin reductases isozymes, TR1, TR2 and TR3, have been recently isolated from mouse tissues and human cDNA sequences for these isozymes were determined (17). We have compared homology among human and C. elegans thioredoxin reductases. TR-Se is 58.5% identical with human TR1, 56.1% with human TR2, and 49.8% identical with human TR3. On the other hand, TR-S is 45.1% identical with human TR1, 45.1% with human TR2 and 48.4% with human TR3. These values are indicative of the close evolutionary relationship between TR-Se, TR1 and TR2, while TR-S is most homologous with TR3. Thus, it is tempting to speculate that TR-Se in C. elegans may have a function similar to that of TR1 and/or TR2, while the function of mammalian TR3 may be maintained in worms by TR-S.

In contrast to $^{75}$Se-labeling of mammalian cells, which detects multiple selenoproteins, $^{75}$Se-labeling of C. elegans detected only a single selenoprotein, TR-Se (Figs. 3 and 4). Other selenoproteins may potentially occur in C. elegans but be present in much lower levels than TR-Se. The recently reported complete sequence of the C. elegans genome (29) provides an opportunity to determine if other selenoprotein genes are present in this organism. Hence, we searched the C. elegans genome (29) for homologs of mammalian Sec-containing proteins. In addition to thioredoxin reductase genes, we identified the C. elegans genes that encode proteins homologous to mammalian selenoproteins glutathione peroxidase, selenophosphate synthetase 2, Selenoprotein W and the 15 kDa selenoprotein (not shown). However, these homologs contain Cys in place of Sec in mammalian sequences.

Mammalian selenoprotein genes contain a SECIS element in the 3'-untranslated region (3'-UTR) of the corresponding mRNAs (30,31) and all mammalian thioredoxin reductase genes contain such a 3'-UTR motif (17). Previously identified non-mammalian eukaryotic selenoprotein, glutathione peroxidase from trematode Schistosoma mansoni (32), contained mammalian-type SECIS element, which was also present in the 3'-UTR. These SECIS elements consist of a conserved ATGAN(10-12 nucleotides)AAN(15-27 nucleotides)NGAN nucleotide sequence. In the SECIS stem-loop structure TGAN forms essential non-Watson-Crick base pairing with NGAN sequence, and AAN is located in the apical loop or bulge (Fig. 5). The SECIS element is necessary for recognition of UGA as a codon for Sec rather than a signal for termination of translation (31) and mutations in the conserved quartet nucleotides resulted in abolishing Sec incorporation (33).
We examined the 3′UTR of the TR-Se gene and found putative structures (not shown) that are similar to mammalian SECIS elements and that also satisfy the energy requirements for such mRNA structures. However, these structures lacked the ATGA consensus sequence characteristic of a mammalian SECIS element. In addition, we found a structure in the coding region (nucleotides 1410-1475) (Fig. 5) that fits to the previously proposed mammalian SECIS structure (31,33). Hence, one can envision that Sec in TR-Se is inserted by either a SECIS element in the coding region upstream of the Sec-inserting TGA codon, or it is inserted by structures that are present in 3′UTR, but do not have a canonical SECIS element. Although the presence of a SECIS element is a highly rare event in mRNA sequences, the occurrence of this SECIS-like structure may be fortuitous.

We further tested the C. elegans genome for the presence of mammalian-type SECIS elements with the program (25) that identifies SECIS elements in human selenoprotein genes and found no matches to the regions that corresponded to coding regions in exons. If a putative SECIS element that was detected in the coding region of TR-Se is a functional structure, the lack of direct SECIS matches during these computer searches favors the notion that TR-Se may be a single selenoprotein in C. elegans. It should be noted, however, that further experimental analyses are necessary to demonstrate whether the putative SECIS in the coding region, or other mRNA structures in the 3′UTR (or both) are required for Sec incorporation.

If TR-Se is indeed the only selenoprotein in C. elegans, the complex system for Sec incorporation, which consists of a SECIS element, Sec tRNA, selenophosphate synthetase, Sec synthase and the specific Sec region, or other mRNA structures in the 3′UTR, but do not have a canonical SECIS element. Although the presence of a SECIS element is a highly rare event in mRNA sequences, the occurrence of this SECIS-like structure may be fortuitous.

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If TR-Se is indeed the only selenoprotein in C. elegans, the complex system for Sec incorporation, which consists of a SECIS element, Sec tRNA, selenophosphate synthetase, Sec synthase and the specific Sec translation factor, must be maintained by C. elegans exclusively for inserting Sec into this enzyme. Nature presumably utilizes the Sec residue in thioredoxin reductases to take advantage of the ionization and redox properties of selenium. Thus, the present studies suggest an essential role of the C-terminal penultimate Sec residue in TR-Se function. Further experiments directly addressing the functional role of C. elegans thioredoxin reductase, including knockout studies of the TR-Se and/or the Sec tRNA genes, should provide solutions to these issues.

REFERENCES