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Identification of a protein component of a mammalian tRNA^{Sec} complex implicated in the decoding of UGA as selenocysteine

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ABSTRACT

This report describes a novel RNA-binding protein, SECp43, that associates specifically with mammalian selenocysteine tRNA (tRNA^{Sec}). SECp43, identified from a degenerate PCR screen, is a highly conserved protein with two ribonucleoprotein-binding domains and a polar/acidic carboxy terminus. The protein and corresponding mRNA are generally expressed in rat tissues and mammalian cell lines. To gain insight into the biological role of SECp43, affinity-purified antibody was employed to identify its molecular partners. Surprisingly, the application of native HeLa cell extracts to a SECp43 antibody column results in the purification of a 90-nt RNA species identified by direct sequencing and Northern blot analysis as tRNA^{Sec}. The purification of tRNA^{Sec} by the antibody column is striking, based on the low abundance of this tRNA species. Using recombinant SECp43 as a probe for interacting protein partners, we also identify a 48-kDa interacting protein, which is a possible component of the mammalian selenocysteine insertion (SECIS) pathway. To our knowledge, SECp43 is the first cloned protein demonstrated to associate specifically with eukaryotic tRNA^{Sec}.

Keywords: mammalian SECIS pathway; protein synthesis; RNA-binding protein; selenocysteine insertion; selenocysteine tRNA

INTRODUCTION

The nonstandard amino acid selenocysteine is an integral component of selenium-containing enzymes (Cone et al., 1976; Stadtman, 1996). Crystal structure analysis reveals selenocysteine in the enzyme active site, which implicates its role in the mechanism of the reaction (Ladenstein et al., 1979). The functional advantage of this residue is supported by site-directed mutagenesis studies in which a reduction of enzymatic activity results from the substitution of a cysteine codon in place of a codon for selenocysteine (Axley et al., 1991; Berry et al., 1992). The insertion of selenocysteine occurs cotranslationally as specified by an in-frame UGA codon (Chambers et al., 1986; Zinoni et al., 1986). More commonly, an in-frame UGA codon signals termination, which presents the interesting dilemma of how the translational machinery distinguishes these two meanings of UGA in mRNAs.

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What is the mechanism by which selenocysteine is incorporated into the polypeptide chain? As selenocysteine-containing proteins are found in all three kingdoms, to what extent are the mechanistic features inherent in one line of decent conserved in another? Current evidence supports a model in which the recognition of UGA as a selenocysteine codon, rather than a stop codon, is dependent upon the presence of mRNA secondary structures termed selenocysteine insertion sequence (SECIS) elements (Heider et al., 1992; for review see Stadtman, 1996; Low & Berry, 1996; Atkins et al., 1999). SECIS elements are RNA stem-loop structures found immediately downstream of the selenocysteine-specific UGA codon in bacterial mRNAs, whereas, in mRNAs of eukaryotes and archaebacteria, SECIS elements are located in the 3′ untranslated region, frequently at a considerable distance from the selenocysteine codon.

An outline of the biochemical pathway for selenocysteine insertion in bacteria has been elucidated by genetic and biochemical studies in which the products of the *sel*A, *sel*B, *sel*C, and *sel*D genes are shown to be required for the insertion of selenocysteine at the di-

rection of SECIS elements (Leinfelder et al., 1988a, 1988b, 1989; Forchhammer et al., 1989, 1990). A key feature of this pathway involves the function of a specialized selenocysteine tRNA ($tRNA^{Sec}$; product of the *sel*C gene), which is initially charged with serine and subsequently converted to selenocysteyl-$tRNA^{Sec}$ (Baron & Böck, 1995). In correspondence to these factors, only $tRNA^{Sec}$ and the selenophosphate synthetase gene (homolog of *sel*D) have been identified in eukaryotes (Lee et al., 1989; Low et al., 1995).

An important unanswered question is how, for eukaryotes, do the SECIS elements in the 3′ untranslated region signal, at a distance, the cotranslational incorporation of selenocysteine at UGA codons? In particular, an understanding of the molecular link between $tRNA^{Sec}$ and SECIS RNA elements has remained elusive, in part, because the eukaryotic homolog of SELB has not yet been identified. In a model proposed by Ringquist et al. (1994) it is held that SELB delivers bacterial selenocysteyl-$tRNA^{Sec}$ to a ribosome-SECIS RNA complex. This model implicitly accounts for the recognition of a selenocysteine codon by coupling the required SECIS RNA recognition event to the delivery of the charged tRNA. In eukaryotes, the known elongation factor, EF-1α, fails to deliver selenocysteyl-$tRNA^{Sec}$ to the ribosome, and a partially characterized factor is implicated for this role (Jung et al., 1994; Yamada, 1995).

Recent progress has been made in the identification and cloning of a mammalian protein, dbpB, which recognizes SECIS RNA elements (Shen et al., 1998). Nonetheless, a variety of proteins that have been detected in association with mammalian SECIS RNA elements, or by autoimmune antibodies that recognize $tRNA^{Sec}$–protein complexes, have yet to be fully characterized (Gelpi et al., 1992; Shen et al., 1995; Hubert et al., 1996). The lack of cloned forms of these factors hampers progress in understanding their individual contributions to the mechanism of selenocysteine insertion. In the present study, we report the cloning of a novel rat RNA-binding protein, SECp43, and identify its specific RNA ligand as $tRNA^{Sec}$. In addition, we demonstrate a specific interaction between SECp43 and a 48-kDa protein partner, the latter of which may be a previously identified component of the SECIS insertion machinery. SECp43 and its related antibody should prove useful reagents in further studies aimed at elucidating the mechanism of selenocysteine insertion in mammals.

RESULTS

Sequence and expression of a novel RNA-binding protein, SECp43

In a screen for RNA-binding proteins enriched in neural tissue, a novel open reading frame was identified with characteristics of an RNA-binding domain (RBD) and further investigated. To identify the corresponding full-length cDNA, the cloned DNA fragment containing the RBD was used as a probe to screen a cDNA library derived from rat cerebellum. A 1.1-kb cDNA clone was identified and determined by sequence analysis to contain an open reading frame of 861 bp (Fig. 1). The RBD identified in the initial screen was found internal to the 1.1-kb cDNA, and a second RBD was found immediately upstream in the same reading frame. A region enriched in polar and acidic residues was found in the carboxy terminal half of the predicted protein sequence.

To test for mRNA expression, the DNA fragment used to screen the cDNA library was used as a probe in a Northern blot analysis with poly A^+ RNA derived from rat tissues, as well as cell lines of rat and human origin. Ubiquitous expression of SECp43 mRNA, 1.3 kb in length, with some variation relative to control β-actin mRNA is evident (Fig. 2A).

To test for protein expression, a specific antibody to SECp43 was generated to a histidine-tagged recombinant protein (see Materials and Methods). The SECp43 antibody was affinity purified prior to Western blot analysis. A single band of an apparent molecular weight of 43 kDa is the major reactive species observed in nuclear (NE) and cytoplasmic (S100) extracts of HeLa cells (Fig. 2B, lanes NE and S100).

A 90-nt RNA identified as $tRNA^{Sec}$ copurifies with SECp43 protein from native HeLa cell extracts

The presence of two RNA-binding domains in the SECp43 protein sequence suggests that this protein might associate with RNA in vivo. To identify candidate RNA partners, SECp43 protein was immunoaffinity purified under native conditions from HeLa extracts using a column containing covalently-linked SECp43 antibody. Briefly, a total cell lysate from HeLa cells (CSK) was applied to the antibody column and eluted under low pH conditions. Eluted fractions were deproteinized and labeled with ^{32}pCp and T4 RNA ligase. These results show that a 90-nt RNA species, which is larger than bulk tRNA, is specifically copurified on the anti-SECp43 column, compared to a control column containing covalently linked bovine serum albumin (BSA) (Fig. 3A, lanes 1 and 2, arrow). In addition to the CSK extract, HeLa S100 and nuclear extracts gave similar results (fig. 3A, lanes 3–6). These results show that affinity purification of the 90-nt RNA, (RNA X), is reproducible for different native extract preparations and that RNA X is enriched in the cytoplasmic extract.

To determine the identity of RNA X, the affinity purification experiment was scaled up to obtain enough RNA for direct enzymatic sequencing from the labeled 3′ end. The digestion pattern of the gel-purified RNA X

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                                                        G TCT GGC CGC
CGA GGC AGC GTG TAC GCA GGA GGT GCC TCA GGC GCG GGC TCG GCT GCG CAG
GCT GGC GCG GTT GCG GGC CAC AGG CCC GCA CGG TCC CCG ACC GGG TGC GGG

^
ATG GCG GCC AGC CTC TGG ATG GGA GAC CTG GAA CCC TAC ATG GAT GAG AAC       51
Met Ala Ala Ser Leu Trp Met Gly Asp Leu Glu Pro Tyr Met Asp Glu Asn       17     RNP2(1)

TTC ATC TCT AGA GCC TTT GCC ACC ATG GGG GAG ACC GTG ATG AGT GTC AAA      102
Phe Ile Ser Arg Ala Phe Ala Thr Met Gly Glu Thr Val Met Ser Val Lys       34

ATT ATC CGA AAC CGC CTC ACT GGA ATC CCA GCT GGC TAC TGC TTT GTG GAA      153
Ile Ile Arg Asn Arg Leu Thr Gly Ile Pro Ala Gly Tyr Cys Phe Val Glu       51     RNP1(1)

TTC GCA GAC TTG GCC ACA GCC GAG AAG TGT TTG CAT AAA ATT AAT GGG AAA      204
Phe Ala Asp Leu Ala Thr Ala Glu Lys Cys Leu His Lys Ile Asn Gly Lys       68

CCC CTT CCG GGA GCC ACA CCT GCA AAA CGT TTT AAA CTG AAT TAT GCC ACT      255
Pro Leu Pro Gly Ala Thr Pro Ala Lys Arg Phe Lys Leu Asn Tyr Ala Thr       85

TAC GGG AAA CAG CCA GAC AAC AGC CCT GAG TAC TCC CTG TTT GTG GGG GAC      306
Tyr Gly Lys Gln Pro Asp Asn Ser Pro Glu Tyr Ser Leu Phe Val Gly Asp      102     RNP2(2)

CTG ACC CCA GAC GTG GAC GAT GGC ATG CTG TAT GAG TTC TTT GTC AAA GTC      357
Leu Thr Pro Asp Val Asp Asp Gly Met Leu Tyr Glu Phe Phe Val Lys Val      119

TAC CCC TCC TGC CGG GGA GGC AAG GTG GTT TTG GAC CAG ACA GGC GTG TCT      408
Tyr Pro Ser Cys Arg Gly Gly Lys Val Val Leu Asp Gln Thr Gly Val Ser      138

AAG GGC TAT GGC TTT GTG AAA TTC ACA GAT GAG TTG GAG CAG AAG CGA GCC      459
Lys Gly Tyr Gly Phe Val Lys Phe Thr Asp Glu Leu Glu Gln Lys Arg Ala      153     RNP1(2)

TTG ACG GAG TGC CAG GGA GCA GTG GGA CTG GGG TGC AAG CCT GTG CGG CTG      510
Leu Thr Glu Cys Gln Gly Ala Val Gly Leu Gly Cys Lys Pro Val Arg Leu      170

AGT GTG GCC ATC CCC AAA GCG AGC CGT GTA AAG CCA GTT GAG TAC AGC CAG      561
Ser Val Ala Ile Pro Lys Ala Ser Arg Val Lys Pro Val Glu Tyr Ser Gln      187

ATG TAC AGT TAC AGC TAC AAC CAG TAT TAC CAG CAG TAC CAG AAC TAC TAT      612
Met Tyr Ser Tyr Ser Tyr Asn Gln Tyr Tyr Gln Gln Tyr Gln Asn Tyr Tyr      204

GCC CAG TGG GGC TAT GAC CAG AAC ACT GGC AGC TAC AGC TAC AGC TAC CCT      663
Ala Gln Trp Gly Tyr Asp Gln Asn Thr Gly Ser Tyr Ser Tyr Ser Tyr Pro      221

CAG TAT GGC TAT ACC CAG AGC ACC ATG CAG ACA TAT GAA GAG GTT GGG GAT      714     polar/
Gln Tyr Gly Tyr Thr Gln Ser Thr Met Gln Thr Tyr Glu Glu Val Gly Asp      238     acidic

GAT GCA CTG GAA GAC CCT GCG CCA CAG CTG GAT GTG ACT GAG GCC AAC AAG      765
Asp Ala Leu Glu Asp Pro Ala Pro Gln Leu Asp Val Thr Glu Ala Asn Lys      253

GAG TTC ATG GAA CAG AGT GAG GAA CTG TAC GAT GCA CTG ATG GAC TGT CAC      816
Glu Phe Met Glu Gln Ser Glu Glu Leu Tyr Asp Ala Leu Met Asp Cys His      272

TGG CAG CCC CTG GAT ACC GTG TCA TCA GAG ATC CCC GCC ATG ATG TAG CCA      861
Trp Gln Pro Leu Asp Thr Val Ser Ser Glu Ile Pro Ala Met Met ***          287

GGA CAG AGC ACA GAT GCT TTT TAT TGT GAA TCT TTT ATC TTT TTG AAA GTG
ATG TAT AAT GTC TTA ATA AAT TAC CAT TTC CTG AAA AAA AAA AAA AAA AAA
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FIGURE 1. cDNA sequence and deduced amino acid sequence of SECp43 (GenBank accession number AF181856). The cDNA clone is 1.1 kb in length and contains an open reading frame of 861 bp. Two RNA-binding domains found at the amino terminus of the protein are indicated by the presence of conserved RNP1 and RNP2 sequences (underlined). A polar/acidic domain at the carboxy terminus is indicated.

gave unique sequence information, which was used in a blast N search of the GenBank database and found to match human tRNA^{Sec} (Fig. 3B). The mouse, bovine, and human tRNA^{Sec} sequences are identical (Diamond & Dudock, 1981; Kato et al., 1983). The secondary structure of tRNA^{Sec} is shown in Figure 3C with the bold type representing the positions identified by the direct sequencing of RNA X.

To verify that RNA X is tRNA^{Sec}, the gene for human tRNA^{Sec} was cloned by polymerase chain reaction (PCR) and used as a probe in a Northern blot analysis. These results clearly show that the probe for tRNA^{Sec} hybridizes specifically with the 90-nt RNA eluted from the anti-SECp43, but not the control column (Fig. 4, lanes 5, 6). The DNA probe is highly specific as indicated by a single hybridizing RNA of 90 nt for a sucrose gradient fraction containing the majority of bulk tRNA (Fig. 4, lane 4). A single band is also observed when total RNA from HeLa cells is probed (data not shown).

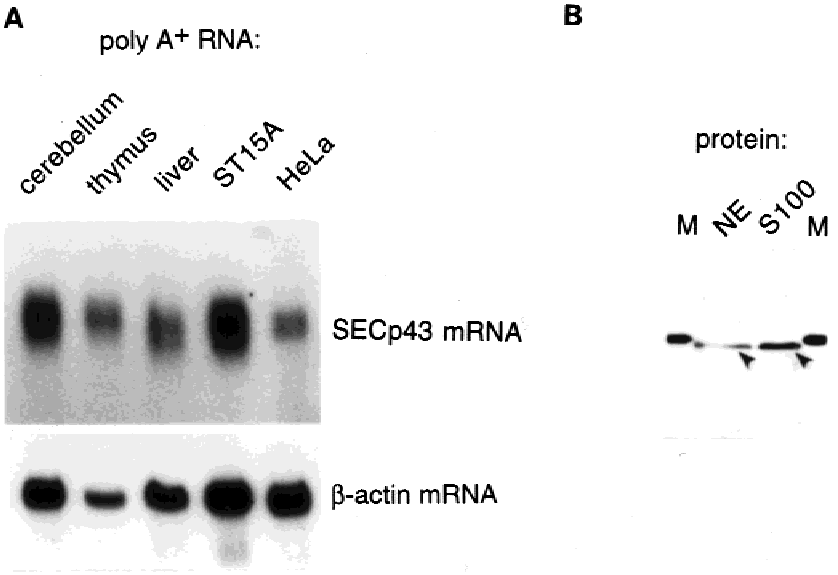

FIGURE 2. Expression of SECp43 mRNA and protein. **A**: Northern blot analysis of poly A^+ RNA purified from rat cerebellum, thymus, liver, and cell lines corresponding to rat cerebellum (ST15A) and HeLa. Top panel is a Northern blot with a DNA probe corresponding to the second RBD of the SECp43 clone. Bottom panel represents the same blot hybridized with a β-actin mRNA probe. **B**: Western blot analysis of SECp43 protein. An affinity-purified chicken polyclonal antibody raised against the recombinant SECp43 protein was used to test for protein expression in 100 μg samples of HeLa cytoplasmic (S100) and nuclear (NE) extracts. Arrowhead indicates major protein species detected in each extract. M lanes represent samples of recombinant SECp43 protein containing a 6× histidine tag at the amino terminus, which confers a slower mobility.

Recombinant SECp43 specifically blocks association of $tRNA^{Sec}$ with the antibody column

Because the anti-SECp43 antibody used in the affinity selection experiment of Figure 3A was raised against a histidine-tagged recombinant protein, we considered the possibility that some fraction of the antibody might recognize the histidine tag. To test this possibility, the experiment was repeated in the presence of excess recombinant SECp43 and U2 snRNP auxiliary factor large subunit (U2AF) proteins, each of which contain an identical N-terminal histidine tag. In this experiment, the competitor protein was preincubated with the column resin and washed with CN buffer prior to the application of extract. Both pCp labeling and Northern blot analysis were used to assay the recovered RNA samples. These results clearly show that recombinant SECp43 effectively blocks $tRNA^{Sec}$ from associating with the antibody column, whereas U2AF has no effect (Fig. 5, lanes 3–6). Thus, we conclude that $tRNA^{Sec}$ associates specifically with SECp43 protein.

Specific interaction of SECp43 with a 48-kDa protein

Experiments to assay direct binding of recombinant SECp43 protein to in vitro transcribed human $tRNA^{Sec}$ have not met with success. This may be explained by the requirement for specific tRNA modifications or for additional cofactors and/or assembly steps not reproduced by these in vitro conditions. To identify candidate SECp43-interacting protein partners, protein overlay analysis was used to probe ammonium sulfate fractions from HeLa S100 extract using ^{32}P-labeled SECp43 protein. These results show that SECp43 interacts with a 48-kDa protein partner in the 20–50% ammonium sulfate fractions (Fig. 6, lanes 2–4). A control blot demonstrates the specificity of the interaction, such that binding to ^{32}P-labeled SECp43 is lost in the presence of a 200-fold molar excess of unlabeled SECp43 (Fig. 6, lanes 5, 6).

DISCUSSION

Here we identify a novel RNA-binding protein, SECp43, which associates with mammalian $tRNA^{Sec}$. Copurification of $tRNA^{Sec}$ and SECp43 is striking because the cellular concentration of $tRNA^{Sec}$ is much lower than that of bulk tRNA. Association is demonstrated by the selection of $tRNA^{Sec}$ from native HeLa cell lysates using affinity purified SECp43 antibody. Enzymatic sequencing and Northern blot analysis provide compelling evidence that the RNA coimmunopurified with SECp43 is a unique species and identical to mature $tRNA^{Sec}$. Importantly, immunoaffinity purification of $tRNA^{Sec}$ depends upon the SECp43 epitope. The competition experiment of Figure 5 rules out the possibility that epitopes other than SECp43 are responsible for purification of $tRNA^{Sec}$, because excess recombinant SECp43 but not a control protein with the same affinity tag blocks selection of the tRNA by the antibody column.

SECp43 has a modular structure in which the amino terminal half of the protein contains two RNA-binding domains, whereas the carboxy terminal half contains a polar/acidic region. The gene is highly conserved in mammals, suggesting an essential function. That is, mouse and human homologs in the EST database show $\geq$92% sequence identity throughout the length of the cDNA. More distant relationships are suggested by protein database searches. The amino terminal half of SECp43 shows homology to the RNA-binding protein

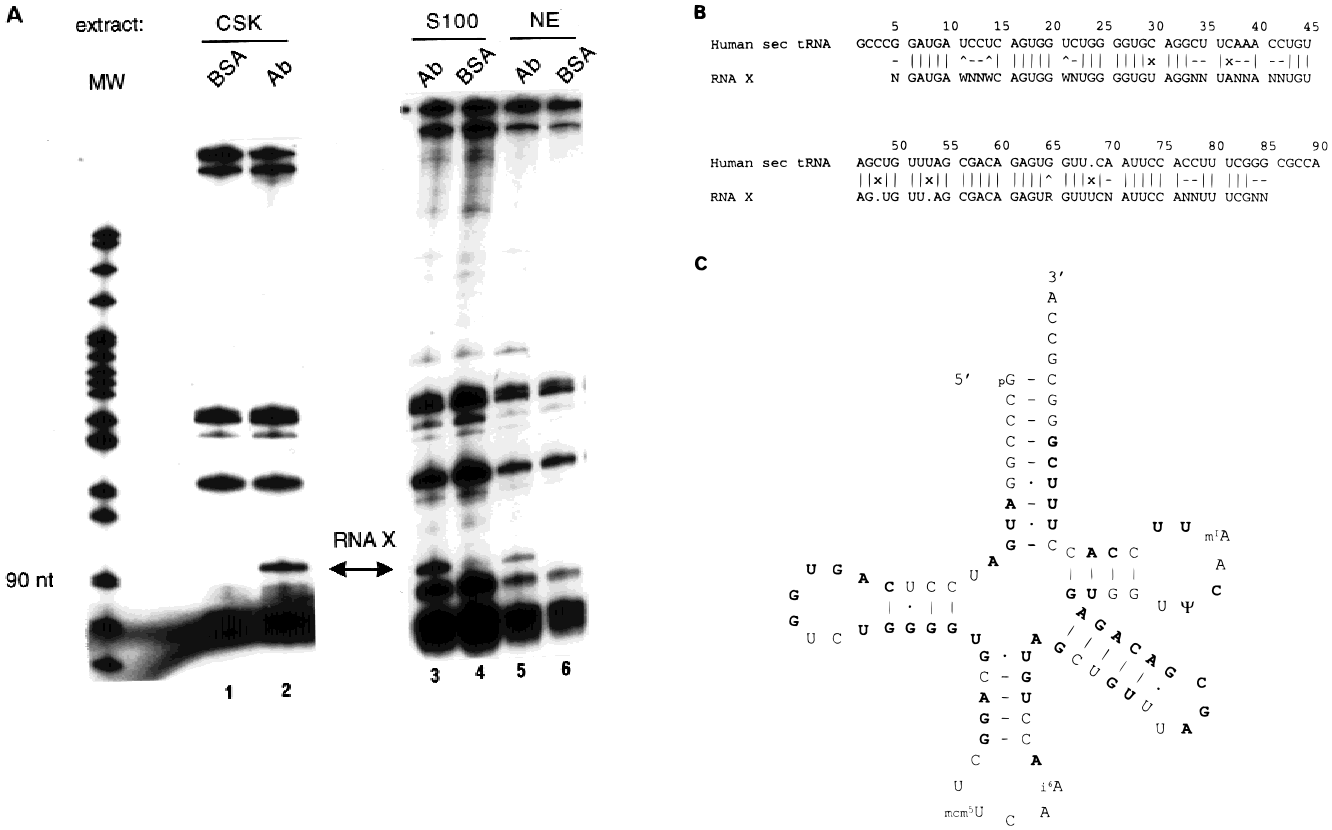

FIGURE 3. Immunoaffinity selection of SECp43 protein demonstrates copurification of tRNA^{Sec} from native HeLa cell extracts. **A**: A SECp43-specific antibody column was generated by covalent linkage of affinity-purified antibody to sepharose beads (lanes Ab). Control columns were generated with bovine serum albumin (lanes BSA). Three types of native extracts from HeLa cells were applied to the columns: Triton X-100 extract (CSK), cytoplasmic (S100), and nuclear (NE). Subsequent to low pH elution and deproteinization, the resulting RNA samples were ^{32}pCp labeled and resolved on 10% polyacrylamide/7 M urea gels. RNA X is indicated by arrowheads in lanes 2, 3, and 5. **B**: Linear representation of human tRNA^{Sec} (top) and RNA X (bottom). Lines: matches; x: mismatches; arrowheads: ambiguous positions. **C**: Secondary structure of tRNA^{Sec} is shown according to the suggested convention of Hubert et al. (1998). Bold residues determined by direct enzymatic sequencing of RNA X match exactly to the sequence and position in the tRNA. Modified residues are likely to be responsible for the difficulty in obtaining sequence information in the anticodon loop and portion of the T loop.

CSX1 of the fission yeast, *Schizosaccharomyces pombe* (44% identical residues in a region of 177 amino acids). In *Saccharomyces cerevisiae*, homology to two RNA-binding proteins, Nam8p and polyadenylate binding protein, is evident (39% and 29% identical residues, respectively), but again this homology is limited to the RNA-binding module of SECp43. Selenocysteine insertion is unknown in yeast; consequently, these similarities may simply reflect evolutionary relatedness of the RNA-binding region of SECp43.

SECp43 is one of the rare examples of a protein with ribonucleoprotein (RNP) motifs that is shown to associate specifically with a tRNA. RNP motifs, also termed RNA recognition motifs, contain characteristic RNP1 octamer and RNP2 hexamer sequences, and are commonly found in proteins that bind pre-mRNA, mRNA, rRNA, and snRNA (Kenan et al., 1991; Burd & Dreyfuss, 1994). Many members of this diverse protein family function in RNA processing or transport. Thus, the two RNP domains found in the SECp43 clone prompted us to predict that the protein has a natural RNA ligand, and this prediction was confirmed in our experiments. In eukaryotes, one other protein shown to contain an RNP domain and bind to a form of tRNA is the La autoantigen. La protein binds to pre-tRNA in the location of the 3′ trailer where it is believed to influence tRNA end processing by facilitating the formation of correctly folded pre-tRNA (Wolin & Matera, 1999). It is likely that pre-tRNA^{Sec} binds to La protein in its earliest "nuclear" stage; however, there is no evidence to indicate that La is structurally or functionally related to SECp43. Nor is it likely for La to be in the same RNP complex as SECp43, as SECp43 is bound only to mature tRNA^{Sec} and is predominantly cytoplasmic.

The specific association of SECp43 with tRNA^{Sec} points to the hypothesis that SECp43 plays a role in the mechanism of selenocysteine insertion. SECp43 protein is not related to seryl aminoacyl-tRNA synthetase or to any of the known factors identified in the bacterial pathway that converts seryl-tRNA^{Sec} to selenocysteyl-

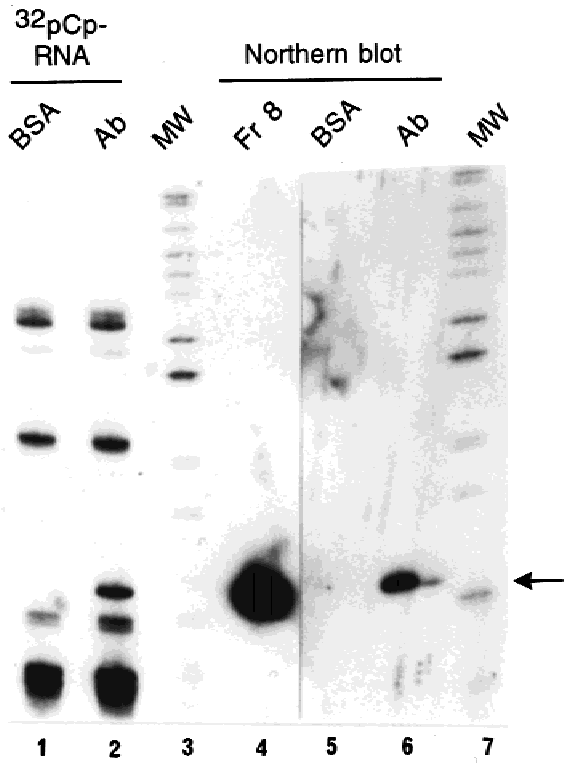

FIGURE 4. Northern blot analysis verifies that tRNA^{Sec} is the 90-nt RNA that copurifies with SECp43. A human tRNA^{Sec}-specific probe was generated by PCR and used to probe a blot containing RNA fractions from the specific antibody column (lane 6) or control BSA column (lane 5). Arrowhead marks the position of the 90-nt RNA X. A sucrose gradient fraction of HeLa S100 extract (lane Fr 8) contains bulk tRNA. Ab and BSA column samples identical to those used in lanes 5 and 6 were labeled with ^{32}pCp as shown at left (lanes 1,2). Lanes MW: pBR322 *Msp*I digest.

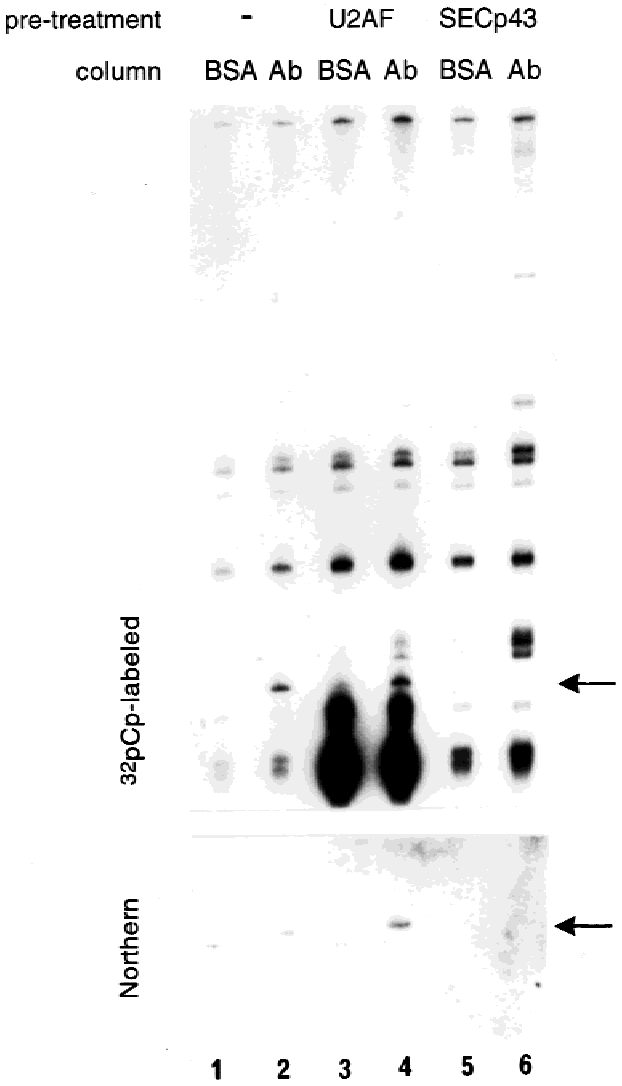

FIGURE 5. Immunoaffinity selection of tRNA^{Sec} is blocked in the presence of excess recombinant SECp43, but not by a control RNA-binding protein. Top panel: ^{32}pCp-labeled RNA species eluted from specific antibody columns (Ab) or control BSA columns (BSA). The columns were pretreated with buffer (lanes −), excess control protein (U2AF), or excess SECp43 as indicated (top). Bottom panel: Northern blot of samples of top panel performed with a DNA probe specific for tRNA^{Sec}. Arrowheads mark position of tRNA^{Sec}.

tRNA^{Sec}. Nor is SECp43 related to the recently identified eukaryotic SECIS recognition protein dbpB, which contains arginine-rich motifs (Shen et al., 1998). However, SECp43 might interact with dbpB (see below). Here we show that immunoaffinity purification of tRNA^{Sec} is reproducible with different preparations of native HeLa cell extracts, consistent with a model in which tRNA^{Sec} and SECp43 are associated in a complex in vivo and this complex is retained during the immunoaffinity selection procedure.

To explore the nature of the SECp43 interaction with tRNA^{Sec}, attempts were made to assemble the complex with an in vitro-synthesized tRNA^{Sec} transcript. The purified recombinant SECp43 protein, either in the presence or absence of HeLa extract, is not capable of assembling into a complex containing synthetic tRNA^{Sec} (F. Ding & P.J. Grabowski, unpubl. observations). Others have reported that the T7 transcript of tRNA^{Sec} can be charged with serine by seryl-tRNA synthetase (Amberg et al., 1996; Heckl et al., 1998); however, subsequent conversion to selenocysteyl-tRNA^{Sec} is not achieved in vitro (Watanabe et al., 1997). It is most likely that SECp43 is one of those factors that fails to associate with a T7 transcript of tRNA^{Sec} because of the requirement for specific modification of the tRNA and/or assembly events not reproduced by our in vitro conditions. Another possibility is that a charged form of tRNA^{Sec} is required for association of SECp43. Thus, the present study does not distinguish whether SECp43 interacts directly or indirectly with tRNA^{Sec}.

Based on the experiment of Figure 6, it is interesting to speculate that SECp43 might contribute to the delivery of selenocysteyl-tRNA^{Sec} to the ribosome at UGA codons in response to SECIS RNA elements. An understanding of how SECIS elements direct selenocysteine insertion at UGA codons suffers from the lack of available (cloned) factors that physically connect SECIS

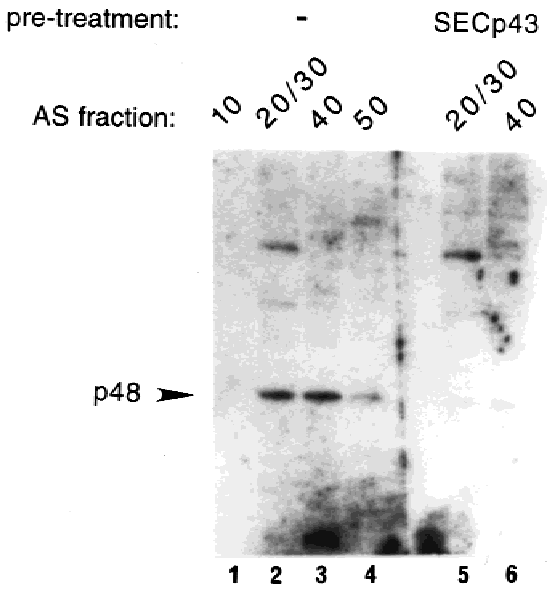

FIGURE 6. Protein overlay analysis identifies a 48-kDa interacting protein partner of SECp43. Lanes 1–4: 10–50% ammonium sulfate fractions (AS) of HeLa S100 extract were assayed with a ^{32}P-labeled version of recombinant SECp43 as indicated (top). Parallel blots were treated with aliquots of the same labeled SECp43 probe without (lanes 1–4), or with (lanes 5,6) excess, unlabeled SECp43. Arrowhead: SECp43 interacting p48 protein.

elements and charged tRNA^{Sec}. SECp43 might function as such an adaptor. Here, protein overlay analysis demonstrates a specific association of recombinant SECp43 with a 48-kDa protein species in HeLa S100 extracts. It is likely that the 48-kDa SECp43-interacting protein observed in the present study is identical to the 48-kDa antigen detected by patient autoantibodies, which was shown in a previous report to immunoprecipitate tRNA^{Sec} (Gelpi et al., 1992). We have attempted to test this possibility; however, the patient antibodies are no longer available. It is also intriguing to consider the suggestion of Shen et al. (1998) that the SECIS-interacting protein dbpB might be the same as the 48-kDa autoantigen reported in the Gelpi et al. (1992) study. If the 48-kDa protein observed in the present study is the same as that reported in these previous studies, this would point to an adaptor role for SECp43 in the mechanism of selenocysteine insertion. An alternative possibility is that the 48-kDa protein observed in the experiment of Figure 6 is a molecular bridge that supports the interaction of tRNA^{Sec} and SECp43. SECp43 and its related antibody should prove to be useful reagents to test these ideas.

MATERIALS AND METHODS

Degenerate RT-PCR and cDNA cloning

Poly A$^+$ RNA was isolated from rat granule neurons and reverse transcribed with random primers. Degenerate primers corresponding to the highly conserved RNP I and RNP II sequences were designed based on a subset of mammalian hnRNP proteins, similar to the method of Kim & Baker (1993). Degenerate primers contained the following sequences (5′ to 3′): GN2, TATATAGAGCTCTSTTYRTSGGNAAYYT; and GN4, ATATATAAGCTTRAAYTCSACRAADSC. PCR mixtures, 100 μL total volume, contained 2.5 ng cDNA, degenerate primers (10 μM each), 200 nM deoxynucleoside triphosphates, 2 mM $MgCl_2$, and 1 μL Taq polymerase in reaction buffer (Perkin Elmer). Conditions used for thermal cycling were: 95 °C, 3 min; cycles 1–5: 94 °C, 30 sec; 48 °C, 30 sec; and 72 °C, 30 sec; cycles 6–40: 94 °C, 30 sec; 55 °C, 30 sec; 72 °C, 30 sec; followed by 72 °C, 10 min. PCR products were purified and cloned into pBS vector (Stratagene). Plasmids containing inserts with open reading frames of the expected size were selected as probes to screen a rat cerebellum cDNA library (Stratagene). In the degenerate screen, approximately 300 plasmids containing the correct insert size were obtained from 10^3 plasmids. Of the 300 inserts selected from the initial screen, DNA sequencing revealed three unique open reading frames, of which SECp43 is represented.

Recombinant protein expression, antibody production, Western blot analysis

The open reading frame of SECp43 cDNA was amplified by PCR and cloned into the *Bam*H1 site of the pQE8 bacterial expression vector (Qiagen), which produces the recombinant protein with a 6× histidine tag at the amino terminus. Recombinant protein was expressed in *Escherichia coli* and purified by metal chelate affinity chromatography on Talon columns (Clontech). Imidazole, 100 mM in 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5, 8 M urea, was used for protein elutions. Recombinant protein was dialyzed in phosphate buffered saline and used to generate polyclonal antibodies in chickens (Cocalico Biologicals). The antibody was affinity purified using a column with immobilized recombinant SECp43 (AminoLink, Pierce). For Western blotting, affinity purified antibody was used at 1:4,000; secondary antibody was anti-chicken IgY-HRP (Promega) at 1:25,000.

Cell extract preparation

HeLa spinner cells were grown to a density of 3–5 × 10^5 cells/mL in Joklik's minimal essential medium with 5% horse serum (Sigma). CSK extracts were prepared essentially as described (Fey et al., 1986). Briefly, cells were washed in cold phosphate-buffered saline. The packed-cell pellet was resuspended in 10 vol cold CSK buffer (10 mM PIPES, pH 6.8, 3 mM $MgCl_2$, 100 mM NaCl, 300 mM sucrose, 1 mM EGTA) with 0.5% Triton X-100, protease inhibitors (22.5 μg/mL aprotonin, 15 μg/mL benzamidine, 5 μg/mL leupeptin, 22.5 μg/mL soybean trypsin inhibitor), and 4 mM vanadyl ribonucleotide complex (Gibco BRL) as ribonuclease inhibitor. Insoluble nuclear matrix was removed by centrifugation at 650 × *g*, 4 °C, 5 min. The supernatant was collected and stored at −80 °C (CSK extract). Dignam nuclear and cytoplasmic (S100) extracts were prepared as described by Dignam et al. (1983), except that D buffer contained 5% glycerol (final concentration).

Immunopurification of SECp43

Antibody resin (Ab-resin) was prepared as follows: purified chicken anti-SECp43 antibody, 2.8 mg, was conjugated to 2 mL of Aminolink Plus activated sepharose (Pierce). Control resin (BSA-resin) was prepared in parallel with 4 mg of bovine serum albumin. HeLa CSK extract, 500 μL, Dignam nuclear, or S100 extract (100 μL) were centrifuged at 12,000 $\times$ *g* for 5 min. The clarified extracts were incubated with 20 μL of packed Ab-resin, or BSA-resin for 1 h at 4 °C with gentle rotation. After centrifugation at 700 $\times$ *g* for 20 s, the supernatants were removed, and the resins washed four times with 1 mL of CN buffer (10 mM PIPES, pH 6.8, 3 mM $MgCl_2$, 100 mM NaCl, 300 mM sucrose, 1 mM EGTA, 0.05% NP40). Columns were eluted with 100 μL of 0.1 M glycine, pH 2.5.

RNA 3′-end labeling and direct sequencing

3′-CMP was phosphorylated in a 25-μL reaction containing 1 μL of 25-mM 3′-CMP, 2.5 μL of 10$\times$ polynucleotide kinase buffer, 1 μL of T4 polynucleotide kinase, 3 μL of γ-[^{32}P]ATP (6,000 Ci/mmol, 150 μCi/μL) at 37 °C for 2 h. The enzyme was heat inactivated, 100 °C, 1 min. Purified RNA was dissolved in 8.3 μL of water and labeled with 1.7 μL of [^{32}P]pCp mix, 2 μL of 10$\times$ T4 RNA ligase buffer, 1.7 μL of dimethylsulfoxide, 3.4 μL of 50% glycerol, and 3.0 μL of T4 RNA ligase at 4 °C, overnight. Labeled RNA was purified by phenol/chloroform extraction and ethanol precipitation. RNA sequencing was performed with RNase T1 (Gp/N), RNase U2 (Ap/N), Phi M (Up/N and Ap/N), and RNase *Bacillus cereus* (Up/N and Cp/N) (kit, Pharmacia). Each reaction contained 6,000–20,000 cpm labeled RNA and 0.1–10 U enzyme.

Protein overlay analysis of SECp43 associating proteins

A heart muscle kinase site (8 amino acids) was introduced at the carboxy terminus of the SECp43 open reading frame by PCR amplification with primers (5′ to 3′): M7–9, CGCGGATC CATGGCGGCCAGCCTCTGGATG; and M7–16, CGCGGAT CCGAATTCAACACTTGCCCTTCTTGCCATCATGGCGGG GATCTC. The PCR fragment was cloned into PQE8 vector and recombinant protein expressed and purified as above. SECp43 protein was labeled at the HMK site as follows: 100 ng of protein was incubated with 25 μCi of γ-^{32}P-ATP (3,000 Ci/mmol), 6 U heart muscle kinase (Sigma) in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$, and 1 mM dithiothreitol (DTT) in a 15-μL volume at 37 °C for 1 h. Unincorporated ATP was removed by gel filtration chromatography on a 5-mL Sephadex G25 column. Protein extracts were resolved on 12.5% polyacrylamide gels and blotted onto PVDF membrane (Immobilon-p, Millipore). All manipulations were performed at 4 °C. Membrane was blocked in 20 mL binding buffer (1% BSA, 20 mM HEPES, pH 7.6, 100 mM NaCl, 0.2 mM EDTA, 3 mM $MgCl_2$, 0.5 mM DTT) for 2 h. Binding was performed in 20 mL binding buffer with 2 $\times$ 10^6 cpm radioactive SECp43 overnight. As controls, unlabeled SECp43 or ATP was mixed with the radioactive probe followed by incubation with the membrane. Membranes were washed in binding buffer 5 $\times$ 20 mL prior to autoradiography.

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REFERENCES

Amberg R, Mizutani T, Wu XQ, Gross HJ. 1996. Selenocysteine synthesis in mammalia: An identity switch from tRNA(Ser) to tRNA(Sec). *J Mol Biol 263*:8–19.

Atkins JF, Böck A, Matsufuji S, Gesteland RF. 1999. Dynamics of the genetic code. In: Gesteland RF, Cech TC, Atkins JF, eds. *The RNA World*, 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 637–673.

Axley MJ, Böck A, Stadtman TC. 1991. Catalytic properties of an *Escherichia coli* formate dehydrogenase mutant in which sulfur replaces selenium. *Proc Natl Acad Sci USA 88*:8450–8454.

Baron C, Böck A. 1995. The selenocysteine-inserting tRNA species: Structure and function. In: Söll D, RajBhandary U, eds. *tRNA: Structure, biosynthesis, and function*. Washington, DC: American Society for Microbiology Press. pp 529–544.

Berry MJ, Maia Al, Kieffer JD, Harney JW, Larsen PR. 1992. Substitution of cysteine for selenocysteine in type I iodothyronine deiodinase reduces the catalytic efficiency of the protein but enhances its translation. *Endocrinology 131*:1848–1852.

Burd CG, Dreyfuss G. 1994. Conserved structures and diversity of functions of RNA-binding proteins. *Science 265*:615–621.

Chambers I, Frampton J, Goldfarb P, Affara N, McBain W, Harrison PR. 1986. The structure of the mouse glutathione peroxidase gene: The selenocysteine in the active site is encoded by the termination codon, TGA. *EMBO J 5*:1221–1227.

Cone JE, del Rio RM, Davis JN, Stadtman TC. 1976. Chemical characterization of the selenoprotein component of clostridial glycine reductase: Identification of selenocysteine as the organoselenium moiety. *Proc Natl Acad Sci USA 73*:2659–2663.

Diamond A, Dudock B. 1981. Structure and properties of a bovine liver UGA suppressor serine tRNA with a tryptophan anticodon. *Cell 25*:497–506.

Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res 11*:1475–1489.

Fey EG, Krochmalnic G, Penman S. 1986. The nonchromatin substructures of the nucleus: The ribonucleoprotein (RNP)-containing and RNA-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. *J Cell Biol 102*:1654–1665.

Forchhammer K, Leinfelder W, Böck A. 1989. Identification of a novel translation factor necessary for the incorporation of selenocysteine into protein. *Nature 342*:453–456.

Forchhammer K, Rücknagel KP, Böck A. 1990. Purification and biochemical characterization of SELB, a translation factor involved in selenoprotein synthesis. *J Biol Chem 265*:9346–9350.

Gelpi C, Sontheimer EJ, Rodriguez-Sanchez JL. 1992. Autoantibodies against a serine tRNA–protein complex implicated in cotranslational selenocysteine insertion. *Proc Natl Acad Sci USA 89*:9739–9743.

Heckl M, Busch K, Gross HJ. 1998. Minimal tRNA(Ser) and tRNA(Sec) substrates for human seryl-tRNA synthetase: Contribution of tRNA domains to serylation and tertiary structure. *FEBS Lett 427*:315–319.

Heider J, Baron C, Böck A. 1992. Coding from a distance: Dissection of the mRNA determinants required for the incorporation of selenocysteine into protein. *EMBO J 11*:3759–3766.

Hubert N, Sturchler C, Westhof E, Carbon P, Krol A. 1998. The 9/4 secondary structure of eukaryotic selenocysteine tRNA: More pieces of evidence. *RNA 4*:1029–1033.

Hubert N, Walczak R, Carbon P, Krol A. 1996. A protein binds the selenocysteine insertion element in the 3′ UTR of mammalian selenoprotein mRNAs. *Nucleic Acids Res 24*:464–469.

Jung JE, Karoor V, Sandbaken MG, Byeong JL, Ohama T, Gesteland RF, Atkins JF, Mullenbach GT, Hill KE, Wahba AJ, Hatfield DL. 1994. Utilization of selenocysteyl-tRNA$^{[Ser]Sec}$ and seryl-tRNA$^{[Ser]Sec}$ in protein synthesis. *J Biol Chem 269*:29739–29745.

Kato N, Hoshino H, Harada F. 1983. Minor serine tRNA containing anticodon NCA (C4 RNA) from human and mouse cells. *Biochem Int 7*:635–645.

Kenan DJ, Query CC, JD Keene. 1991. RNA recognition: Towards identifying determinants of specificity. *Trends Biochem Sci 16*: 214–220.

Kim YJ, Baker BS. 1993. Isolation of RRM-type RNA-binding protein genes and the analysis of their relatedness by using a numerical approach. *Mol Cell Biol 13*:174–183.

Ladenstein R, Epp O, Bartels K, Jones A, Huber R. 1979. Structure analysis and molecular model of the selenoenzyme glutathione peroxidase at 2.8 Å resolution. *J Mol Biol 134*:199–218.

Lee BJ, Worland PJ, Davis JN, Stadtman TC, Hatfield DL. 1989. Identification of selenocysteyl-tRNA(Ser) in mammalian cells that recognizes the nonsense codon, UGA. *J Biol Chem 264*:9724–9727.

Leinfelder W, Forchhammer K, Zinoni F, Sawers G, Mandrand-Berthelot MA, Böck A. 1988a. *Escherichia coli* genes whose products are involved in selenium metabolism. *J Bacteriol 170*:540–546.

Leinfelder W, Stadtman TC, Böck A. 1989. Occurrence in vivo of selenocysteyl-tRNA$_{UCA}^{Ser}$ in *Escherichia coli*: Effect of *sel* mutations. *J Biol Chem 264*:9720–9723.

Leinfelder W, Zehelein E, Mandrand-Berthelot MA, Böck A. 1988b. Gene for a novel tRNA species that accepts *L*-serine and cotranslationally inserts selenocysteine. *Nature 331*:723–725.

Low SC, Berry MJ. 1996. Knowing when not to stop: Selenocysteine incorporation in eukaryotes. *Trends Biochem Sci 21*:203–208.

Low SC, Harney JW, Berry MJ. 1995. Cloning and functional characterization of human selenophosphate synthetase, an essential component of selenoprotein synthesis. *J Biol Chem 270*:21659–21664.

Ringquist S, Schneider D, Gibson T, Baron C, Böck A, Gold L. 1994. Recognition of the mRNA selenocysteine insertion sequence by the specialized translation elongation factor SELB. *Genes & Dev 8*:376–385.

Shen Q, McQuilkin PA, Newburger PE. 1995. RNA-binding proteins that specifically recognize the selenocysteine insertion sequence of human cellular glutathione peroxidase mRNA. *J Biol Chem 270*:30448–30452.

Shen Q, Wu R, Leonard JL, Newburger PE. 1998. Identification and molecular cloning of a human selenocysteine insertion sequence-binding protein: A bifunctional role for DNA-binding protein B. *J Biol Chem 273*:5443–5446.

Stadtman TC. 1996. Selenocysteine. *Annu Rev Biochem 65*:83–100.

Watanabe T, Kanaya K, Fujiwara T, Mizutani T. 1997. Preliminary investigation of tRNA modification enzymes with Se in bovine liver. *Nucleic Acids Symp Ser 37*:155–156.

Wolin SL, Matera AG. 1999. The trials and travels of tRNA. *Genes & Dev 13*:1–10.

Yamada K. 1995. A new translational elongation factor for selenocysteyl-tRNA in eukaryotes. *FEBS Lett 377*:313–317.

Zinoni F, Birkmann A, Stadtman TC, Böck A. 1986. Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from *Escherichia coli. Proc Natl Acad Sci USA 83*:4650–4654.