MOLECULAR AND CELLULAR BIOLOGY, Apr. 1997, p. 1977–1985 Vol. 17, No. 4
0270-7306/97/$04.00+0

An RNA-Binding Protein Recognizes a Mammalian Selenocysteine Insertion Sequence Element Required for Cotranslational Incorporation of Selenocysteine

ANDREA LESOON,† ANURADHA MEHTA, RAJNISH SINGH, GUY M. CHISOLM, AND DONNA M. DRISCOLL*

Department of Cell Biology, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

Received 18 September 1996/Returned for modification 21 November 1996/Accepted 23 January 1997

In mammalian selenoprotein mRNAs, the recognition of UGA as selenocysteine requires selenocysteine insertion sequence (SECIS) elements that are contained in a stable stem-loop structure in the 3′ untranslated region (UTR). In this study, we investigated the SECIS elements and cellular proteins required for selenocysteine insertion in rat phospholipid hydroperoxide glutathione peroxidase (PhGPx). We developed a translational readthrough assay for selenoprotein biosynthesis by using the gene for luciferase as a reporter. Insertion of a UGA or UAA codon into the coding region of luciferase abolished luciferase activity. However, activity was restored to the UGA mutant, but not to the UAA mutant, upon insertion of the PhGPx 3′ UTR. The 3′ UTR of rat glutathione peroxidase (GPx) also allowed translational readthrough, whereas the PhGPx and GPx antisense 3′ UTRs did not. Deletion of two conserved SECIS elements in the PhGPx 3′ UTR (AUGA in the 5′ stem or AAAAC in the terminal loop) abolished readthrough activity. UV cross-linking studies identified a 120-kDa protein in rat testis that binds specifically to the sense strands of the PhGPx and GPx 3′ UTRs. Direct cross-linking and competition experiments with deletion mutant RNAs demonstrated that binding of the 120-kDa protein requires the AUGA SECIS element but not AAAAC. Point mutations in the AUGA motif that abolished protein binding also prevented readthrough of the UGA codon. Our results suggest that the 120-kDa protein is a significant component of the mechanism of selenocysteine incorporation in mammalian cells.

Selenoproteins that contain the nonstandard amino acid selenocysteine (Sec) have been identified in both prokaryotes and eukaryotes (reviewed in references 20 and 30). Many of the selenoproteins are redox enzymes that contain one Sec residue at their active site. These include the formate dehydrogenases; the type 1 (D1), 2, and 3 iodothyronine deiodinases; the family of glutathione peroxidases (GPx); and phospholipid hydroperoxide glutathione peroxidase (PhGPx). A notable exception is selenoprotein P, which contains up to eight Sec residues and is believed to function in the transport of selenium in mammals (13).

The incorporation of Sec into proteins is a unique cotranslational event in which a UGA codon is recognized as a codon for Sec insertion rather than as a signal for translation termination (35). Mutational analyses of the formate dehydrogenase mRNA in *Escherichia coli* have identified specific nucleotide sequences within a stable stem-loop structure immediately 3′ to the UGA codon that are required for Sec incorporation (12, 36). Biochemical and genetic studies have shown that several other factors are necessary for selenoprotein biosynthesis in bacteria (reviewed in reference 5). Three of the gene products (SELA, SELC, and SELD) are required for the synthesis of $tRNA^{sec}$, a tRNA that is aminoacylated with Sec and contains the anticodon UCA. The fourth gene product, SELB, is directly involved in the decoding of UGA as Sec. SELB is a specialized elongation factor that is highly homologous to EF-Tu and IF2 and binds specifically to $tRNA^{sec}$ (10). SELB also binds to specific sequences within the stem-loop of the selenoprotein mRNA and forms a complex with Sec-$tRNA^{sec}$ (1, 12). This complex, stabilized by the binding of GTP, is thought to present the Sec-aminoacylated tRNA to the A site of the ribosome to facilitate incorporation of Sec into the nascent protein (1, 12).

Considerably less is known about the mechanism of Sec incorporation in eukaryotic cells. A human $tRNA^{sec}$ has been isolated which represents the homolog of the bacterial *selC* gene product (19). Mammalian homologs of the bacterial selenophosphate synthetase, SELD, have also been identified (17, 21). In spite of these similarities, mammalian selenoproteins cannot be expressed in bacteria (24), which suggests that the Sec translation machinery is not completely conserved between prokaryotes and eukaryotes. The recognition of UGA as Sec in mammalian selenoprotein mRNAs requires selenocysteine insertion sequence (SECIS) elements that consist of several short, conserved nucleotide sequences within a stable stem-loop structure (2). The characterized SECIS motifs include three consecutive, unpaired A residues in the terminal loop; AUGA in the stem 8 to 10 nucleotides (nt) 5′ of the terminal loop; and UGR in the 3′ region of the stem across from the AUGA element (2, 3, 27). In contrast to bacteria, the mammalian SECIS elements are located within the 3′ untranslated region (UTR) rather than immediately downstream of the UGA codon in the coding region (2). Because the distance between the stem-loop and the UGA codon varies from 500 to 2,000 nt in selenoprotein mRNAs, it is likely that the mammalian mechanism of Sec insertion involves a component(s) that mediates long-range interactions between the upstream UGA codon and the 3′ UTR. There is no evidence of a mammalian homolog of SELB that might direct Sec insertion at a UGA codon by binding to a SECIS element(s) in the 3′ UTR. A candidate region for RNA-protein interactions is the 5′

* Corresponding author. Mailing address: Department of Cell Biology, Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, NC-10, Cleveland, OH 44195. Phone: (216) 445-9758. Fax: (216) 444-9404.

† Present address: Scriptgen Pharmaceuticals, Inc., Medford, MA 02155.

TABLE 1. Oligonucleotides used in this study

Enzyme and oligonucleotide designations	Sequence[a] (explanation)
Rat PhGPx	
RS1	CAGTGAATTCCCTACAAGTGTTGGCC (nt 655–671)
RS2	GCTAAAGCTTCCCACAAGGCAGCCA (complementary to nt 838–854)
AB4	TCCACCCCGGCACTC****CGGTCTGCCTGAAAACAG (nt 711–748; deletion of ATGA)
AB4A	CGGCACTCACGACGGTCTG (nt 718–736; conversion of ATGA to ACGA)
AB4B	CGGCACTCATGCCGGTCTGCC (nt 718–738; conversion of ATGA to ATGC)
AB4C	CGGCACTCATGGCGGTCTGCC (nt 718–738; conversion of ATGA to ATGG)
AB4D	CGGCACTCATCACGGTCTGCC (nt 718–738; conversion of ATGA to ATCA)
AB5	CATGACGGTCTGCCTG*****CAGCCCGCTGGTGGG (nt 725–760; deletion of AAAAC)
AB7	GCAGTCCCGAGGACC***CGTGCATCCCCGCCG (nt 761–793; deletion of TGG)
Rat GPx	
GPX1	CTAAGGAATTCCTGGTATCTGG (nt 953–975)
GPX2	CTTTAAGCTTAGTGGTGAAACCG (complementary to nt 1126–1149)
Luciferase	
AB1	GCAATTGTTCAAGGAACCAGG (nt 369–389; conversion of Gly-38 to TGA)
AB1/TAA	GCAATTGTTTAAGGAACCAGGGC (nt 369–388; conversion of Gly-38 to TAA)
AB3	GAATTTCGTCACAGCTGAATACAG (nt 1928–1952; insertion of *Pvu*II site at nt 1935)
AB8	CAAGAAGGGCGGAAAGTC (nt 1893–1910)
AB9	CCTGCCACTCATCGCAG (complementary to nt 1967–1983)

[a] Deletions are represented by asterisks. Nucleotides that differ from the wild-type sequence are underlined.

AUGA SECIS element which has recently been shown to form a novel secondary structure motif involving non-Watson-Crick base pairs (32).

The present study investigated the mechanism of Sec insertion in mammalian cells by using the selenoperoxidase PhGPx as a model. We found that the PhGPx 3′ UTR is sufficient to allow translational readthrough of a UGA codon in a heterologous gene. We also identified conserved sequence motifs in the PhGPx 3′ UTR that are required for readthrough of UGA. Furthermore, we report here that a 120-kDa protein in rat testis binds specifically to the PhGPx 3′ UTR and that binding requires the AUGA SECIS element in the 5′ portion of the stem-loop structure. Mutations in this region that abolish binding of the 120-kDa protein also prevent readthrough of the UGA codon. Our data suggest that the 120-kDa protein is a significant component of the mechanism of Sec incorporation in mammalian cells.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides (Table 1) were purchased from GenoSys Biotechnologies, Inc. (Woodlands, Tex.). The numbers in parentheses in Table 1 correspond to nucleotide positions in the coding strand of the rat PhGPx cDNA (31), the rat GPx cDNA (14), or luciferase plasmid pGL2-Control (Promega), unless indicated otherwise. Oligonucleotides RS1, RS2, GPX1, and GPX2 were used for PCR amplification, AB1 to AB7 were used for mutagenesis, and AB8 to AB9 were used for sequencing in pGL2.

Plasmids. Plasmid pGL2-Control contains the full-length cDNA for luciferase (Promega) and is referred to as pGL2. Plasmid pRSV-β-gal contains the β-galactosidase gene (Promega). The rat PhGPx 3′ UTR, from the stop codon to the polyadenylation signal (nt 655 to 854), was cloned into the *Eco*RI-*Hin*dIII sites of pGEM3zf′(+), pAlter-Ex1, and pcDNA1/Amp (InVitrogen). The rat GPx 3′ UTR (nt 953 to 1149) was cloned into the *Eco*RI-*Hin*dIII site of pGEM3zf′(+). The wild-type or mutant rat PhGPx 3′ UTR was subcloned into the *Pvu*II site generated in pGL2 at nt 1935 by standard molecular cloning techniques (26).

Mutagenesis. Mutagenesis of pGL2 was performed to mutate Gly-38 to TGA or TAA and to create a *Pvu*II site at nt 1935 by the Kunkel method (26). Deletion mutants of the rat PhGPx 3′ UTR were derived by the Kunkel method, and point mutations of the rat PhGPx 3′ UTR were made with the Altered Sites II Ex-1 mutagenesis kit (Promega). Clones containing mutations were identified directly by double-stranded DNA sequencing of miniprep DNA by using Sequenase (Amersham).

Transient transfections. Cos-7 cells were plated at 2×10^5/9.5-cm^2 well in DME/F12 medium containing 10% fetal calf serum and 5 ng of Na_2SeO_3 per ml. For selenium depletion experiments, cells were grown in 0.1% fetal bovine serum in the absence or presence of 5 ng of Na_2SeO_3 per ml for 5 days prior to transfection. At 70 to 80% confluency (18 h), 950 ng of test DNA was cotransfected with 50 ng of pRSV-β-gal DNA by using Lipofectamine (Gibco Bethesda Research Laboratories) in accordance with the manufacturer's recommendations. At 48 h posttransfection, cells were harvested for detection of luciferase activity with the Luciferase Assay System (Promega) or β-galactosidase activity with LumiGal 530 Assay Reagent (Lumigen). Extracts were assayed with an ML2250 luminometer (Dynatech). Results are expressed as luciferase activity normalized to β-galactosidase activity.

Synthesis of RNAs and radiolabeled probes. Plasmid DNAs were linearized in the polylinker and transcribed with T7 or SP6 RNA polymerase to generate sense and antisense RNAs, respectively. Radiolabeled RNA probes were synthesized in the presence of [α-^{32}P]UTP and purified as previously described (8). RNA probes contained sequences from the stop codon to the polyadenylation signal of the rat PhGPx (nt 655 to 854) and GPx (nt 953 to 1149) 3′ UTRs.

Preparation of extracts. Extracts were prepared from rat testis tissue as described by Dodson and Shapiro (7), except that the buffers contained leupeptin (50 μg/ml), phenylmethylsulfonyl fluoride (5 μg/ml), and 200 U of RNAguard (Pharmacia) per ml. The total homogenate was centrifuged at 9,000 × *g*, 100,000 × *g*, and 300,000 × *g* to isolate a postmitochondrial supernatant fraction, an S100 cytosolic fraction, and a crude polysomal pellet, respectively (7). Protein concentrations were determined with the Protein Assay Reagent (Bio-Rad).

UV cross-linking assay. Extracts (20 μg) were incubated with 1 ng of ^{32}P-labeled synthetic RNA for 1 h at 30°C in a final volume of 20 μl containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.1 M KCl, 10 μM $MgCl_2$, 250 μg of tRNA per ml, 10 U of RNAguard, and 10% glycerol. Each reaction mixture was treated with UV radiation (Stratalinker 1800) at 254 nm for 10 min in a 96-well tissue culture plate (Costar). The reaction mixture was then treated with RNase A (1 mg/ml) for 30 min at 37°C. Samples were analyzed by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to autoradiography. Figures were generated with ScanWizard Microscan 1.0.5 (MicroTek) and Adobe Photoshop 3.0.5.

RESULTS

Analysis of the rat PhGPx 3′ UTR. The 3′ UTR of PhGPx is highly conserved across species, showing over 80% homology among the rat, human, and pig sequences (Fig. 1A). Based on computer analysis with the program MulFold (16), the rat PhGPx 3′ UTR has the potential to form several different secondary structures similar in thermodynamic stability (data not shown). Recently, Walczak et al. provided experimental evidence that the last three nucleotides of the 5′ AUGA SECIS element are involved in non-Watson-Crick base pairs in human and rat D1, and rat GPx (32). The structure of the rat PhGPx

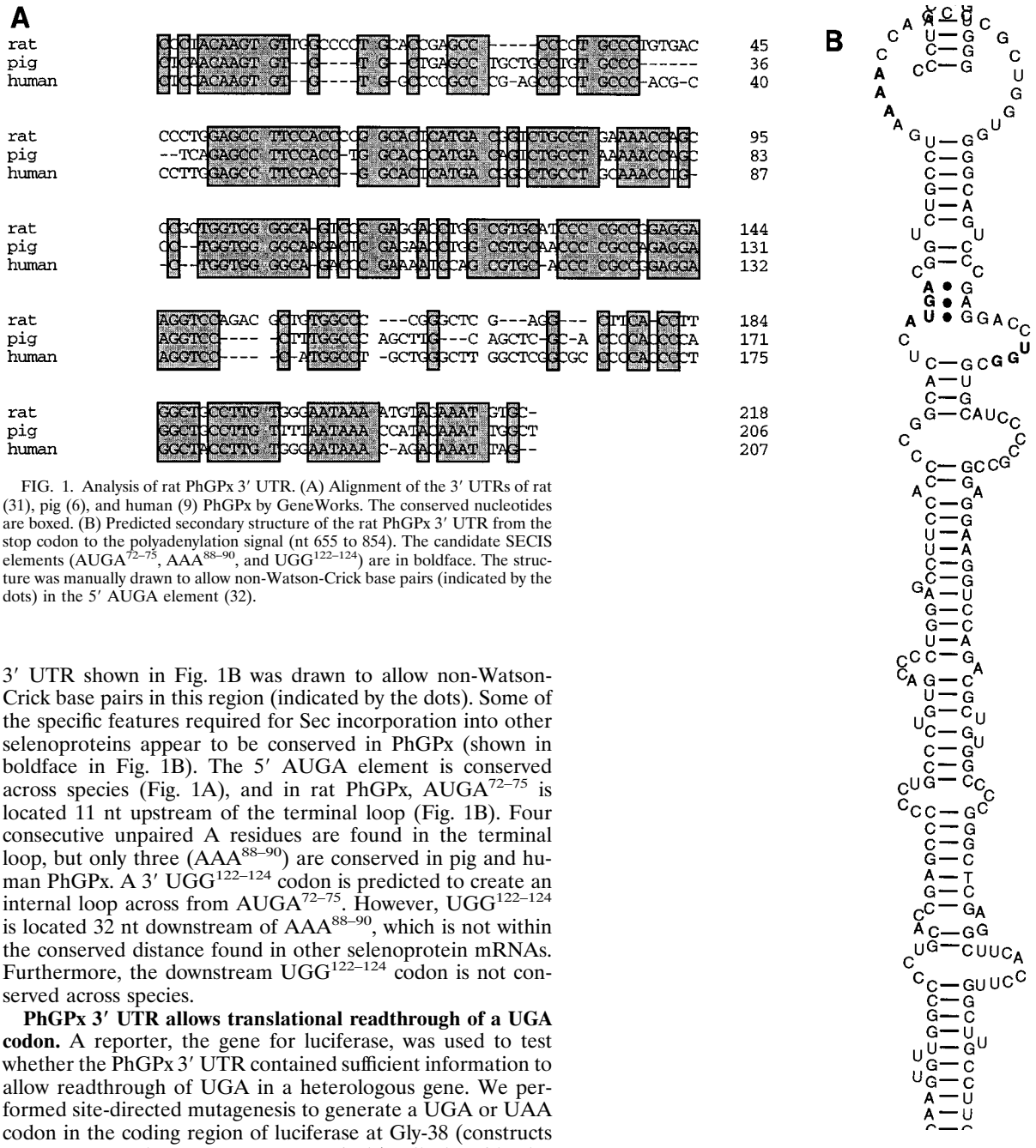

FIG. 1. Analysis of rat PhGPx 3′ UTR. (A) Alignment of the 3′ UTRs of rat (31), pig (6), and human (9) PhGPx by GeneWorks. The conserved nucleotides are boxed. (B) Predicted secondary structure of the rat PhGPx 3′ UTR from the stop codon to the polyadenylation signal (nt 655 to 854). The candidate SECIS elements (AUGA$^{72–75}$, AAA$^{88–90}$, and UGG$^{122–124}$) are in boldface. The structure was manually drawn to allow non-Watson-Crick base pairs (indicated by the dots) in the 5′ AUGA element (32).

3′ UTR shown in Fig. 1B was drawn to allow non-Watson-Crick base pairs in this region (indicated by the dots). Some of the specific features required for Sec incorporation into other selenoproteins appear to be conserved in PhGPx (shown in boldface in Fig. 1B). The 5′ AUGA element is conserved across species (Fig. 1A), and in rat PhGPx, AUGA$^{72–75}$ is located 11 nt upstream of the terminal loop (Fig. 1B). Four consecutive unpaired A residues are found in the terminal loop, but only three (AAA$^{88–90}$) are conserved in pig and human PhGPx. A 3′ UGG$^{122–124}$ codon is predicted to create an internal loop across from AUGA$^{72–75}$. However, UGG$^{122–124}$ is located 32 nt downstream of AAA$^{88–90}$, which is not within the conserved distance found in other selenoprotein mRNAs. Furthermore, the downstream UGG$^{122–124}$ codon is not conserved across species.

PhGPx 3′ UTR allows translational readthrough of a UGA codon. A reporter, the gene for luciferase, was used to test whether the PhGPx 3′ UTR contained sufficient information to allow readthrough of UGA in a heterologous gene. We performed site-directed mutagenesis to generate a UGA or UAA codon in the coding region of luciferase at Gly-38 (constructs pGL2/UGA and pGL2/UAA, respectively); all plasmids also contained the native luciferase 3′ UTR. These constructs were predicted to produce a truncated and therefore inactive protein, since UGA and UAA both function as efficient translation termination codons in mammalian cells (23). The 3′ UTR of PhGPx was then inserted 14 nt downstream of the natural UAA termination codon in luciferase (pGL2/UGA/P and pGL2/UAA/P). The positions of the UGA and UAA codons at Gly-38 were approximately 1,550 nt upstream of the PhGPx 3′ UTR. The luciferase constructs were transiently transfected into selenium-supplemented Cos-7 cells, along with a plasmid encoding β-galactosidase in order to correct for variance in transfection efficiency. As expected, the insertion of a UGA or UAA codon at Gly-38 essentially abolished luciferase activity (Table 2). Upon insertion of the PhGPx 3′ UTR, luciferase activity was restored to a level approximately 24-fold higher than that achieved with pGL2/UGA. This effect of the PhGPx 3′ UTR was codon specific, since an increase in luciferase activity was not detected when cells were transfected with pGL2/UAA/P. As shown in Table 3, readthrough of the UGA codon was selenium dependent since readthrough activity was greatly reduced in selenium-depleted cells compared to selenium-supplemented cells. In addition, we tested the 3′ UTR of another selenoprotein, rat cellular GPx, which also restored luciferase expression (construct pGL2/UGA/G). However, the readthrough activity of the GPx 3′ UTR was only 22% relative to the PhGPx 3′ UTR (Table 2). Insertion of the PhGPx and

TABLE 2. Translational readthrough activities of luciferase reporter constructs

Construct	Codon at Gly-38	Source of 3′ UTR[a]	Avg relative readthrough activity (%)[b] ± SD
pGL2/UGA/P	UGA	PhGPx	100
pGL2/UGA	UGA		4.1 ± 4.3
pGL2/UAA/P	UAA	PhGPx	2.2 ± 1.8
pGL2/UAA	UAA		1.2 ± 0.9
pGL2/UGA/G	UGA	GPx	22.1 ± 6.0
pGL2/UGA/P(AS)	UGA	PhGPx-AS	5.2 ± 2.3
pGL2/UGA/G(AS)	UGA	GPx-AS	4.0 ± 1.4
Deletion mutants			
ΔAUGA	UGA	PhGPx	3.2 ± 1.5
ΔAAAAC	UGA	PhGPx	1.7 ± 0.6
ΔUGG	UGA	PhGPx	160.0 ± 43.1

[a] A TGA or TAA codon was inserted at nt 1935 as described in Materials and Methods. All constructs contained the full-length luciferase 3′ UTR. AS, UTRs in the antisense orientation.

[b] Data represent 3 to 15 independent transfections done as described in Materials and Methods. Results are expressed as readthrough activity relative to that of pGL2/UGA/P, which contains the wild-type PhGPx 3′ UTR.

GPx 3′ UTRs in the antisense orientation did not restore luciferase activity to pGL2/UGA.

Deletion of putative SECIS elements abolishes luciferase activity. We used the luciferase assay to screen mutations in pGL2/UGA/P to identify the sequences in the rat PhGPx 3′ UTR that are required for translational readthrough of UGA. Site-directed oligonucleotide mutagenesis was performed to make deletions of the three candidate SECIS elements, and the deletion mutants were transfected into Cos-7 cells. Deletion of either $AUGA^{72–75}$ or $AAAAC^{87–91}$ abolished luciferase activity (Table 2), suggesting that the particular nucleotide sequences or secondary structures in these regions are important in allowing readthrough. Deletion of $UGG^{122–124}$ did not, however, reduce luciferase activity (Table 2). This finding is consistent with the fact that this sequence is not conserved across species or within the conserved distance from the three consecutive A residues in the terminal loop.

Several proteins bind to the PhGPx and GPx 3′ UTRs. To identify mammalian proteins associated with the incorporation of Sec, we performed UV cross-linking studies with the rat PhGPx and GPx 3′ UTRs. ^{32}P-labeled synthetic RNA transcripts were incubated with cell extracts from rat testis tissue, which expresses high levels of PhGPx (25). The rat testis homogenate was subfractionated by differential centrifugation to produce a postmitochondrial supernatant fraction, an S100 cytosolic fraction, and a 300,000 × *g* polysomal pellet. After UV cross-linking and treatment with RNase A, the samples were analyzed by SDS-PAGE and autoradiography.

TABLE 3. Selenium dependence of translational readthrough activity

Culture condition[a]	Construct	Avg relative readthrough activity (%)[b] ± SD
Selenium supplemented	pGL2/UGA/P	100
	pGL2/UGA	2.5 ± 0.6
Selenium depleted	pGL2/UGA/P	12.5 ± 1.9
	pGL2/UGA	1.3 ± 1.4

[a] Cells were cultured for 5 days prior to transfection in 0.1% fetal bovine serum in the presence (selenium supplemented) or absence (selenium depleted) of 5 ng of Na_2SeO_3 per ml.

[b] Data represent three transfections. Results are expressed as readthrough activity relative to that of pGL2/UGA/P in selenium-supplemented cells.

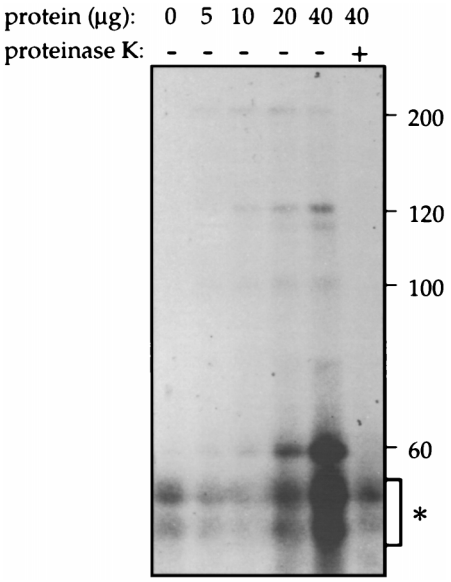

FIG. 2. UV cross-linking of proteins from rat testis tissue to the PhGPx 3′ UTR. UV cross-linking was performed as described in Materials and Methods, with an RNA probe corresponding to the rat PhGPx 3′ UTR from the stop codon to the polyadenylation signal (nt 655 to 854). ^{32}P-labeled RNAs (1 ng) were incubated with increasing amounts of the S100 fraction of a rat testis extract or with protein (40 μg) that was pretreated with proteinase K (1 mg/ml) for 30 min at 37°C prior to incubation. After UV cross-linking and treatment with RNase A, samples were analyzed by SDS–8% PAGE and autoradiography. The positions of the 60-, 100-, 120-, and 200-kDa proteins that were consistently detected are indicated on the right. The asterisk indicates lower-molecular-weight bands associated with the probe. The figure was computer generated by using Scan Wizard Microscan 1.0.5 (MicroTek) and Adobe Photoshop 3.0.5.

As shown in Fig. 2, we identified four proteins with estimated upper molecular masses of 60, 100, 120, and 200 kDa in the S100 fraction which consistently cross-linked to the sense strand of the PhGPx 3′ UTR in a dose-dependent manner. These bands were not detected when the UV cross-linking step was omitted (data not shown). Treatment of the extract with proteinase K abolished all cross-linking, which indicates that these bands represent RNA-protein complexes. In addition, a band below the 120-kDa protein was detected in some experiments. This band may represent a proteolytic breakdown product since it was not consistently detected (compare Fig. 2 and 3). Bands with molecular masses below 60 kDa were detected when the RNA was incubated with buffer only, indicating that these bands were associated with the probe (Fig. 2, asterisk). Subcellular fractionation of the rat testis homogenate indicated that all four proteins were found in the postmitochondrial supernatant (Fig. 3A, lane 2) and the S100 cytosolic fraction (lane 4). The 200-, 120-, and 100-kDa proteins were also detected in the crude polysomal pellet (lane 5), which suggests that these proteins are also polysome associated. Furthermore, the same four proteins also appeared to cross-link to the sense strand of the GPx 3′ UTR (Fig. 3B).

A 120-kDa protein cross-links specifically to the PhGPx 3′ UTR. To assess the specificity of binding of proteins to the ^{32}P-labeled PhGPx 3′ UTR, we performed competition experiments with unlabeled synthetic RNA transcripts. Binding of the 120-kDa protein was significantly reduced by a 10-fold

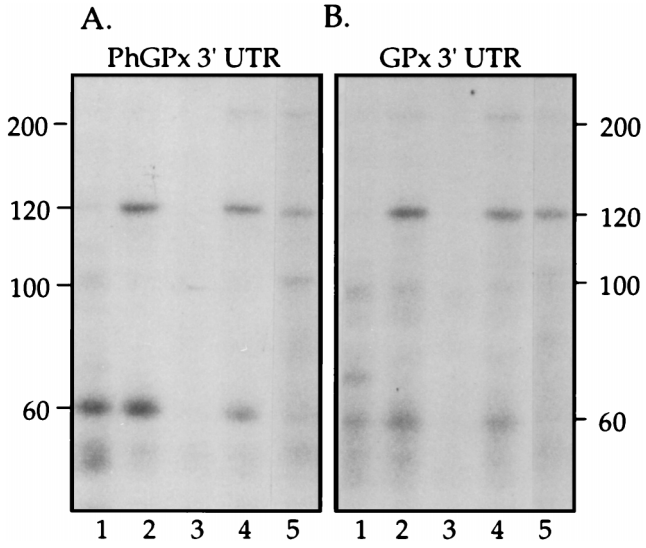

FIG. 3. UV cross-linking of proteins from subcellular fractions of rat testis tissue to selenoprotein 3′ UTRs. A rat testis homogenate was subjected to subcellular fractionation as described in Materials and Methods. RNA probes contained sequences from the stop codon to the polyadenylation signal as described in Materials and Methods. ^{32}P-labeled RNA corresponding to the PhGPx (A) or GPx (B) 3′ UTR was incubated with 20 μg of the total homogenate (lane 1), postmitochondrial supernatant (lane 2), 9,000 × *g* pellet (lane 3), S100 cytosolic fraction (lane 4), or crude polysomal pellet (lane 5). UV cross-linking and SDS-PAGE were performed as described in the legend to Fig. 2. The positions of 60-, 100-, 120-, and 200-kDa proteins are indicated.

molar excess and abolished by a 100-fold molar excess of the wild-type PhGPx 3′ UTR RNA (Fig. 4, lanes 3 to 5). The GPx 3′ UTR RNA was a less efficient competitor, since binding was reduced by a 100-fold excess and eliminated by a 250-fold molar excess of this RNA (lanes 6 to 8). Cross-linking of the 120-kDa protein was not inhibited by an up to 500-fold molar excess of either poly(I · C) RNA (lanes 9 to 11) or tRNA (data not shown). In contrast, the 60-, 100-, and 200-kDa proteins were not significantly competed out by either the PhGPx or the GPx 3′ UTR RNA (lanes 3 to 8). These results suggest that the 60-, 100-, and 200-kDa proteins represent nonspecific RNA binding proteins, whereas the 120-kDa protein may bind to a specific sequence or structure in the 3′ UTRs of selenoprotein mRNAs. In support of this, the 120-kDa protein did not cross-link to antisense transcripts of the PhGPx or GPx 3′ UTR (Fig. 5). The antisense probes did bind to proteins of 60 and 100 kDa, as well as to several other proteins that did not bind to the sense probes (Fig. 5). A 200-kDa protein also cross-linked to the antisense probes, although the signal was fainter than that obtained with the sense probes (data not shown).

We next optimized the incubation conditions to maximize cross-linking of the 120-kDa protein to the ^{32}P-labeled PhGPx 3′ UTR. The optimal KCl concentration was 100 mM, and the optimal temperature was 30°C. Binding was inhibited by $MgCl_2$ concentrations above 1 mM (data not shown). A time course experiment showed that binding of the 120-kDa protein to the probe was not detected at 0 h and required incubation prior to cross-linking (Fig. 6A).

Binding of the 120-kDa protein requires AUGA$^{72–75}$. To determine if binding of the proteins required the putative SECIS elements in the rat PhGPx 3′ UTR, ^{32}P-labeled deletion mutants were analyzed in the cross-linking assay. When the AUGA$^{72–75}$ deletion mutant was used as a probe, the 120-kDa protein was absent (Fig. 7A), even after 3 h of incubation (Fig. 6B). The 120-kDa protein, however, did bind to the AAAAC$^{87–91}$ and UGG$^{122–124}$ deletion mutants (Fig. 7A). Cross-linking of the 60-, 100-, and 200-kDa proteins to each of the mutant RNAs was similar to the pattern generated by the wild-type probe. The results of the direct cross-linking experiments were confirmed by performing competition experiments with the wild-type probe and unlabeled synthetic RNAs corresponding to the deletion mutants. As shown in Fig. 7B, there was no competition for the 120-kDa protein with a 100-fold molar excess of the AUGA$^{72–75}$ deletion mutant RNA (lane 5). Binding of the 120-kDa protein was reduced by a 10-fold, and abolished by a 100-fold, molar excess of the AAAAC$^{87–91}$ deletion mutant RNA (lanes 6 and 7). This mutant was consistently less effective in competition than the wild-type RNA, which suggests that AAAAC is involved in protein recognition. Alternatively, decreasing the size of the terminal loop may alter the structure of the RNA and reduce its affinity for the 120-kDa protein. The UGG$^{122–124}$ deletion mutant (lanes 8 and 9) was an efficient competitor for binding of the 120-kDa protein, which is consistent with our finding that this sequence was not required for translational readthrough in the luciferase reporter assay. None of the deletion mutants competed for binding of the 60-, 100-, and 200-kDa proteins to the wild-type probe (data not shown). These results suggest that the 120-kDa protein requires the conserved 5′ AUGA$^{72–75}$ motif for binding.

The specificity of binding of the 120-kDa protein to the AUGA$^{72–75}$ motif was further assessed by analysis of point mutations in this element. Each point mutant was tested in the cross-linking assay (Fig. 8A) and in the luciferase readthrough assay (Fig. 8B). Two mutations were identified (ACGA and AUCA) that eliminated both cross-linking of the 120-kDa protein and translational readthrough activity. The AUGG mutation resulted in reduced binding of the 120-kDa protein and a

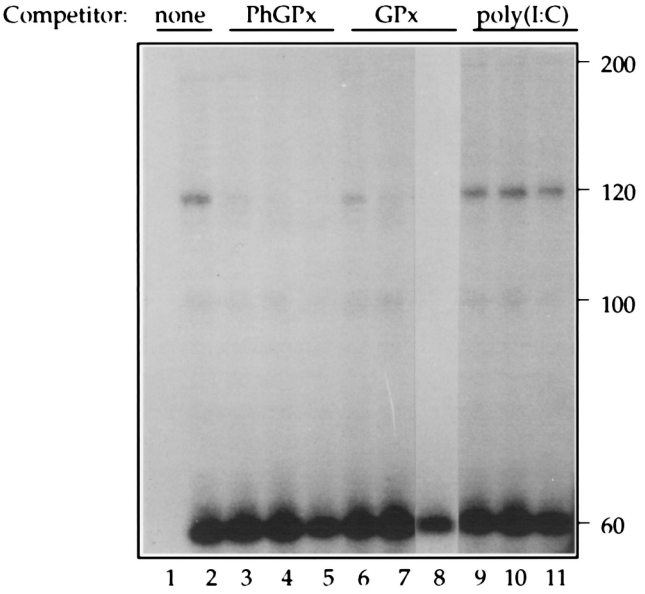

FIG. 4. Competition experiments with unlabeled PhGPx and GPx 3′ UTRs. The ^{32}P-labeled PhGPx 3′ UTR RNA was incubated with no extract (lane 1); 20 μg of the S100 fraction with no competitor (lane 2); 20 μg of the S100 fraction with a 10-, 100-, or 250-fold molar excess of unlabeled PhGPx 3′ UTR RNA (lanes 3 to 5); a 10-, 100-, or 250-fold molar excess of GPx 3′ UTR RNA (lanes 6 to 8); or a 10-, 100-, or 500-fold molar excess of poly(I · C) RNA (lanes 9 to 11). UV cross-linking and SDS-PAGE were performed as described in the legend to Fig. 2. The values on the right are molecular masses in kilodaltons.

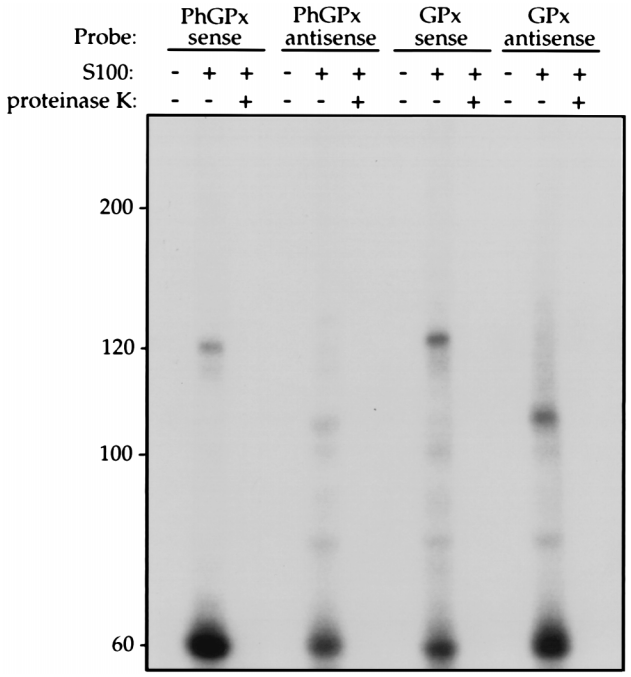

FIG. 5. UV cross-linking experiments with antisense RNAs. RNAs corresponding to the sense and antisense strands of the PhGPx and GPx 3′ UTRs were used as probes. ^{32}P-labeled RNAs were incubated with buffer, 30 μg of the S100 fraction, or 30 μg of the proteinase K-treated S100 fraction as described in the legend to Fig. 2. The positions of the 60-, 100-, 120-, and 200-kDa proteins are shown on the left.

reduction of readthrough activity to 20%. These results demonstrate that binding of the 120-kDa protein is sensitive to single nucleotide changes in the AUGA$^{72-75}$ region and that mutations which disrupt protein binding also eliminate function in the translational readthrough assay. Interestingly, the AUGC mutant retained wild-type levels of protein binding but had no activity in the readthrough assay, which suggests that this mutation disrupts some function other than binding of the 120-kDa protein.

DISCUSSION

The identification of cellular factors and RNA sequences required for the incorporation of Sec into mammalian proteins has been limited by the overall inefficiency of selenoprotein biosynthesis in transfected cells and in vitro translation systems (2, 4). Past studies have relied on sensitive but labor-intensive techniques such as ^{75}Se labeling, affinity labeling, and immunoprecipitation to characterize mammalian SECIS elements (2, 3, 27). In the present study, we developed a UGA readthrough assay which allows us to functionally analyze sequences that are essential for the translation of UGA to Sec in rat PhGPx. This assay utilizes a reporter, the gene for luciferase, which is highly sensitive and linear across several orders of magnitude. While the insertion of Sec was not directly demonstrated in this system, several lines of evidence suggest that the translational readthrough assay is specific for selenoprotein synthesis. The presence of the PhGPx 3′ UTR restored luciferase activity to the UGA mutant but not to the UAA mutant, which demonstrates that readthrough is codon specific. We have shown that translational readthrough of UGA is affected by the selenium content of the medium and is dependent upon the sense orientation of selenoprotein 3′ UTRs. Furthermore, deletion and point mutations of potential SECIS elements in

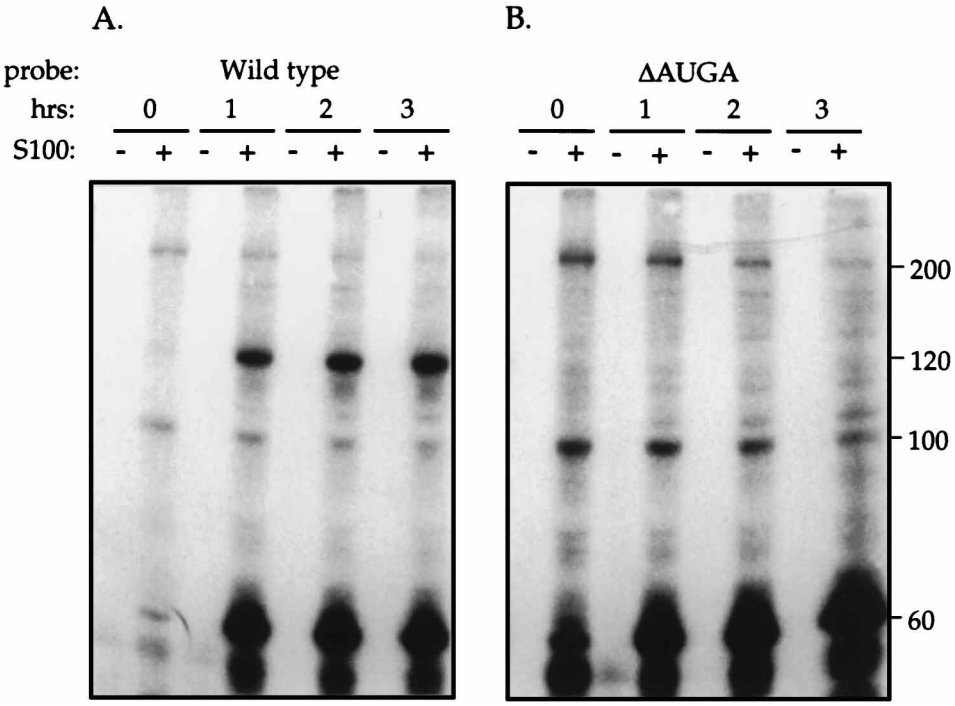

FIG. 6. Time course of protein binding to wild-type and mutant PhGPx 3′ UTRs. ^{32}P-labeled RNAs corresponding to the wild-type PhGPx 3′ UTR (A) or the AUGA$^{72-75}$ deletion mutant (B) were incubated with buffer or 20 μg of the S100 fraction of rat testis extract. Samples were incubated at 30°C for 1, 2, or 3 h, as indicated, prior to UV cross-linking and SDS-PAGE analysis as described in the legend to Fig. 2. The positions of the 60-, 100-, 120-, and 200-kDa proteins are indicated on the right.

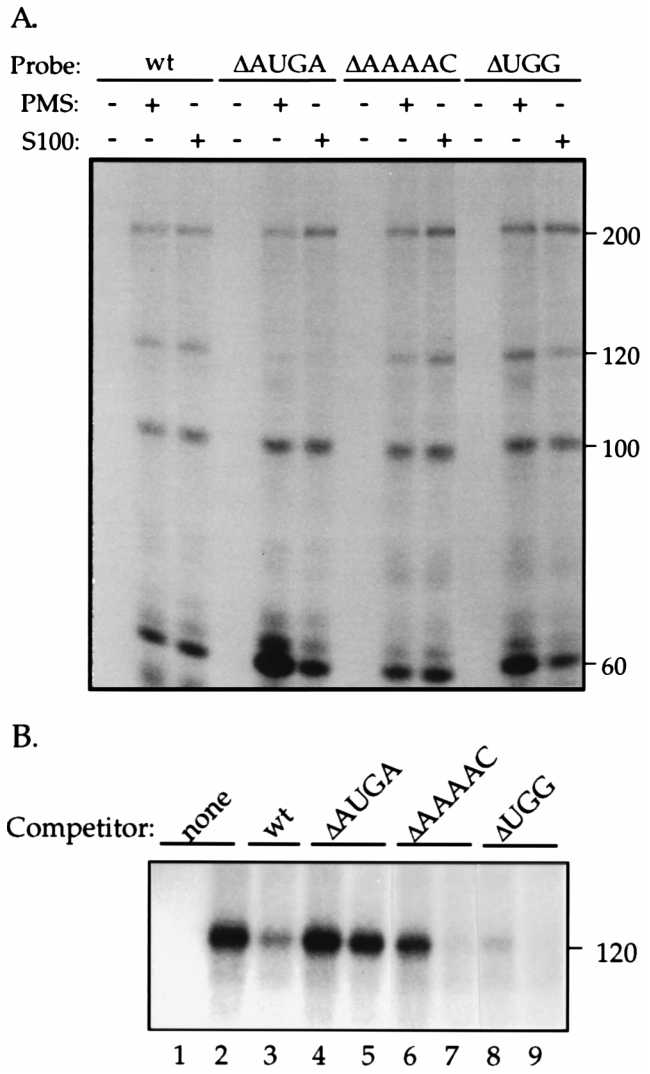

FIG. 7. UV cross-linking experiments with PhGPx deletion mutants. (A) ^{32}P-labeled RNAs corresponding to the wild-type (wt) PhGPx 3′ UTR or the AUGA$^{72–75}$, AAAAC$^{87–91}$, and UGG$^{122–124}$ deletion mutants were incubated with either no extract, 20 μg of the postmitochondrial supernatant (PMS) fraction, or 20 μg of the S100 cytosolic fraction. After UV cross-linking, samples were analyzed by SDS-PAGE as described in the legend to Fig. 2. The positions of 60-, 100-, 120-, and 200-kDa proteins are shown on the right. (B) The ^{32}P-labeled wild-type PhGPx 3′ UTR was incubated with no extract (lane 1); 20 μg of the S100 fraction with no competitor (lane 2); 20 μg of the S100 fraction with a 10-fold molar excess of wild-type (wt) PhGPx 3′ UTR RNA (lane 3); or 10- and 100-fold molar excesses of the AUGA$^{72–75}$ (lanes 4 and 5), AAAAC$^{87–91}$ (lanes 6 and 7), and UGG$^{122–124}$ (lanes 8 and 9) deletion mutant RNAs prior to UV cross-linking. The ability of UGG$^{122–124}$ mutant RNA to compete better than wild-type RNA was not reproducible, and this mutant was equivalent to the wild-type RNA in other competition experiments.

the rat PhGPx 3′ UTR (AUGA$^{72–75}$ and AAAAC$^{87–91}$) abolished readthrough activity. Recently, Kollmus and McCarthy (18) used a similar strategy to demonstrate that the pig PhGPx 3′ UTR suppresses the UGA codon in a β-galactosidase-luciferase fusion gene with an efficiency of readthrough (28-fold above the background) that is similar to ours (24-fold). Both studies effectively show that the PhGPx SECIS element contains sufficient information to permit translational readthrough of a UGA codon in a heterologous gene, as has been reported for the GPx SECIS element (19a, 27). Consistent with these results is the finding that the context of the UGA in the coding region of eukaryotic selenoprotein mRNAs is not an important factor in the decoding of UGA as Sec (22).

Among mammalian selenoproteins, variability exists in the distance (0.5 to 2.0 kb) between the UGA codon for Sec and the SECIS elements in the 3′ UTR. Although the distance from the UGA codon to the 3′ stem-loop structure is approximately 450 nt in the native PhGPx mRNA, the distance in our luciferase construct (1.5 kb) is well within the observed range. More recently, mutagenesis studies have indicated that the distance between the UGA codon and the 3′ UTR can be varied without affecting the efficiency of Sec incorporation but that a minimal distance of 51 to 111 nt is necessary for a UGA codon to be decoded as Sec (22). This spacing requirement may explain why UGA can function as a termination codon in selenoprotein mRNAs when it is immediately upstream of the 3′ UTR (3, 22).

We also identified a novel protein of approximately 120 kDa from rat testis tissue that binds to the sense strands of the 3′ UTRs of PhGPx and GPx. Direct cross-linking experiments and competition studies with mutant RNAs show that binding of the 120-kDa protein is specific and requires AUGA$^{72–75}$ but not AAAAC$^{87–91}$ or UGG$^{122–124}$. This is the first demonstration that one of the conserved mammalian SECIS elements is required for binding of a cellular protein. The 120-kDa protein was not detected in two recent studies that used UV cross-linking to identify proteins in cell extracts that bind to the 3′ UTRs of D1 and GPx (15, 29). This may be due to differences in the source of extract since we used rat testis tissue, which expresses high levels of PhGPx (25). The proteins required for the translation of selenoprotein mRNAs may be more abundant in this tissue. Binding of the 120-kDa protein may also have been inhibited in one of these prior studies by the higher Mg^{2+} concentrations that were used (15).

In addition to demonstrating specificity of binding, we also found strong evidence that the 120-kDa protein is involved in Sec insertion. By using deletion and point mutations of the AUGA$^{72–75}$ motif, we found that mutations that abolished binding of the 120-kDa protein also eliminated function in the translational readthrough assay. It is interesting that the GPx 3′ UTR was a less effective competitor than the PhGPx 3′ UTR for binding of the 120-kDa protein. This result is consistent with our finding that the GPx 3′ UTR was considerably less efficient than the PhGPx 3′ UTR in directing readthrough of a UGA codon. Based on analysis of the selenoprotein P and D1 SECIS elements, Berry and coworkers proposed that the incorporation of Sec may be a point of differential regulation of the synthesis of selenoproteins in vivo (3). Our findings suggest that the PhGPx 3′ UTR has a higher affinity than GPx for the 120-kDa protein or other components of the selenoprotein biosynthetic machinery. The potential differences in affinity may explain the preferential expression of PhGPx over GPx during selenium depletion in vivo (33).

In addition to the 120-kDa protein described here, translation of selenoprotein mRNAs in mammalian cells may require other factors. Several candidate proteins have been proposed, including proteins of 48 and 50 kDa that bind to the aminoacylated form of tRNA^{sec} (11, 34). Although these proteins may represent novel elongation factors homologous to SELB, there is no evidence that they also bind to the SECIS elements in selenoprotein mRNAs. RNA gel shift assays have detected multiple RNA-protein complexes when the human GPx 3′ UTR was incubated with Cos-7 cell extracts (29), and proteins of 55 to 65 kDa that specifically cross-link to the GPx and D1 3′ UTRs have also been reported (15, 29). Although we did not detect these proteins in our system, they may have been obscured by the presence of nonspecific RNA binding proteins

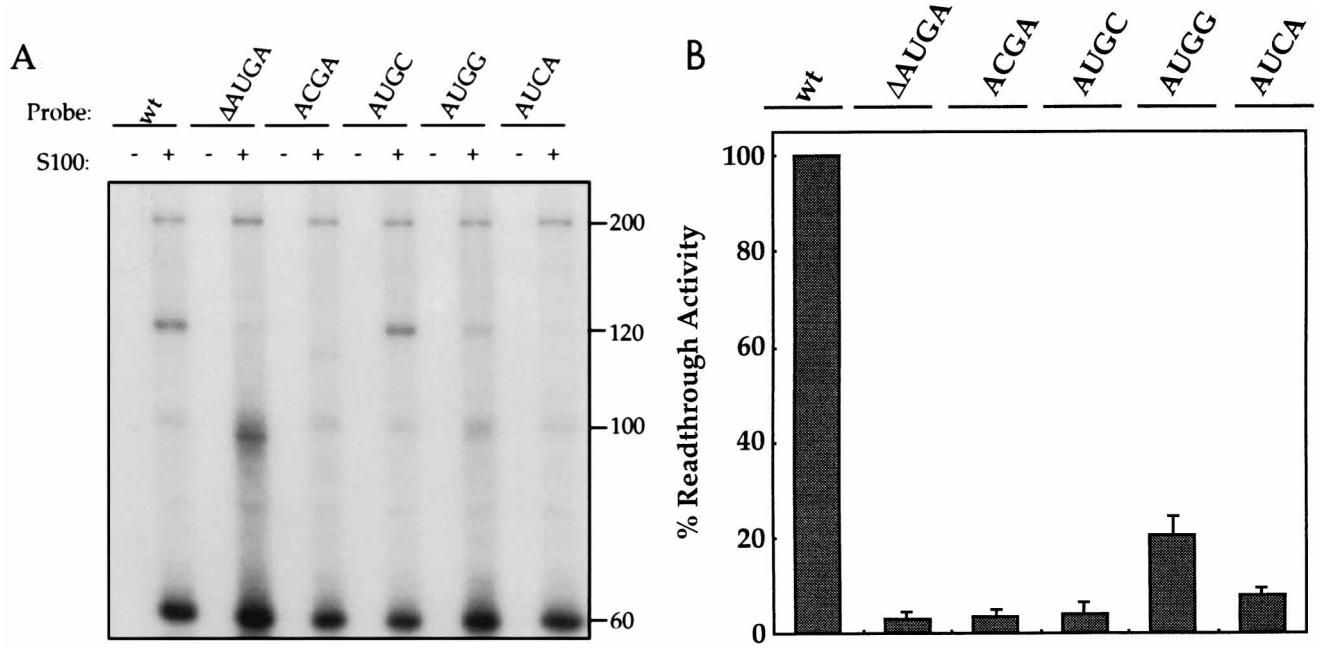

FIG. 8. Analysis of point mutations in AUGA$^{72-75}$. (A) UV cross-linking experiments were performed as described in the legend to Fig. 2, by using ^{32}P-labeled RNAs containing point mutations in AUGA$^{72-75}$. RNAs were incubated with either no extract or 20 μg of the S100 extract. The wild-type (wt) PhGPx 3′ UTR and AUGA$^{72-75}$ deletion mutant (ΔAUGA) RNAs are also shown. The positions of 60-, 100-, 120-, and 200-kDa proteins are shown on the right. (B) The AUGA deletion and point mutants were tested for the ability to allow translational readthrough of a UGA codon in reporter construct pGL2/UGA as described in Table 2. The results, which represent data from four to eight independent transfections, are expressed relative to the wild-type (wt) PhGPx 3′ UTR (100%).

similar in molecular weight in our rat testis extract. Shen et al. (29) demonstrated that binding of the 60- and 65-kDa proteins required the basal stem of the GPx SECIS element, as well as perfect base pairing in this region. However, the role of these proteins in Sec insertion has not been established.

The requirement of AUGA for Sec incorporation has been demonstrated in selenoproteins other than PhGPx (3, 28). The function of the GPx and selenoprotein P AUGA elements has been shown to be highly sensitive to single nucleotide substitutions in this sequence (3, 28), which is consistent with our data. Computer predictions of the secondary structure of selenoprotein 3′ UTRs are ambiguous in the vicinity of the AUGA motif. This region has been predicted to be single stranded in some selenoprotein mRNAs but partially base paired in others (2, 3, 18, 28). Walczak et al. recently determined the secondary structures of the D1 and GPx 3′ UTRs by chemical and RNase probing (32). They proposed that the AUGA motif is part of a novel secondary structure element and that the latter three nucleotides are involved in non-Watson-Crick base pairing. The potential for the formation of a quartet, or at least a triplet, of noncanonical base pairs is conserved among most selenoprotein mRNAs, including rat PhGPx (Fig. 1B). Based on three-dimensional modeling, these non-Watson-Crick base pairs appear to be exposed between an internal loop and an apical helix. Walczak et al. (32) suggested that this element may potentially serve as a site for interaction with either protein or RNA, a model which is supported by our data. Our results demonstrate that AUGA is required for the binding of a 120-kDa protein. However, the actual protein binding site remains to be experimentally determined. Mutagenesis of the putative non-Watson-Crick base pairs in this region may indirectly eliminate protein binding at another site by altering the structure of the RNA. Although the function of the 120-kDa protein was not addressed in our study, it may represent a unique elongation factor analogous to SELB, which binds to tRNA^{sec} and to the stem-loop in selenoprotein mRNAs. The 120-kDa protein could also be an RNA binding protein that facilitates long-range interactions between the upstream UGA and the 3′ UTR. In light of the recent studies of secondary structure, it is conceivable that the 120-kDa protein is an RNA chaperone that modifies the structure or conformation of the RNA to accommodate interaction with a ligand.

ACKNOWLEDGMENTS

We thank Donal Luse, Richard Gronostajski, and Richard Padgett for helpful discussions. We also thank Bruce Carpick for providing poly(I · C) and James Lang for photography.

This research was supported by Public Health Service grants DK 50390 (D.M.D.) and HL 29582 (G.M.C.) from the National Institutes of Health and by an Established Investigatorship Award from the American Heart Association (D.M.D.).

REFERENCES

1. **Baron, C., J. Heider, and A. Böck.** 1993. Interaction of the translation factor SELB with the formate dehydrogenase H selenopolypeptide mRNA. Proc. Natl. Acad. Sci. USA **90:**4181–4185.
2. **Berry, M. J., L. Banu, Y. Chen, S. J. Mandel, J. D. Kieffer, J. W. Harney, and P. R. Larsen.** 1991. Recognition of UGA as a selenocysteine codon in type 1 deiodinase requires sequences in the 3′ untranslated region. Nature **353:** 273–276.
3. **Berry, M. J., L. Banu, J. W. Harney, and P. R. Larsen.** 1993. Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons. EMBO J. **12:**3315–3322.
4. **Berry, M. J., A. L. Maia, J. D. Kieffer, J. W. Harney, and P. R. Larsen.** 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.
5. **Böck, A., K. Forchhammer, J. Heider, and C. Baron.** 1991. Selenoprotein synthesis: an expansion of the genetic code. Trends Biochem. Sci. **16:**463–467.
6. **Brigelius-Flohé, R., K. Aumann, H. Blöcker, G. Gross, M. Kiess, K.-D. Klöppel, M. Maiorano, A. Roveri, R. Schuckelt, F. Ursini, E. Wingender, and L. Flohé.** 1994. Phospholipid-hydroperoxide glutathione peroxidase. Genomic DNA, cDNA, and deduced amino acid sequence. J. Biol. Chem. **269:**7342–7348.

7. **Dodson, R. E., and D. J. Shapiro.** 1994. An estrogen-inducible protein binds specifically to a sequence in the 3′ untranslated region of estrogen-stabilized vitellogenin mRNA. Mol. Cell. Biol. **14:**3130–3138.
8. **Driscoll, D. M., S. Lahke-Reddy, L. M. Oleksa, and D. Martinez.** 1993. Induction of RNA editing at heterologous sites by sequences in apolipoprotein B mRNA. Mol. Cell. Biol. **13:**7288–7294.
9. **Esworthy, R. S., K. Doan, J. H. Doroshow, and F.-F. Chu.** 1994. Cloning and sequencing of the cDNA encoding a human testis phospholipid hydroperoxide glutathione peroxidase. Gene **144:**317–318.
10. **Forchhammer, K., W. Leinfelder, and A. Böck.** 1989. Identification of a novel translation factor necessary for the incorporation of selenocysteine into protein. Nature **342:**453–456.
11. **Gelpi, C., E. J. Sontheimer, and J. L. Rodriguez-Sanchez.** 1992. Autoantibodies against a serine tRNA-protein complex implicated in cotranslational selenocysteine insertion. Proc. Natl. Acad. Sci. USA **89:**9739–9743.
12. **Heider, J., C. Baron, and A. Böck.** 1992. Coding from a distance: dissection of the mRNA determinants required for the incorporation of selenocysteine into protein. EMBO J. **11:**3759–3766.
13. **Hill, K. E., R. S. Lloyd, J.-G. Yang, R. Read, and R. F. Burk.** 1991. The cDNA for rat selenoprotein P contains 10 TGA codons in the open reading frame. J. Biol. Chem. **266:**10050–10053.
14. **Ho, Y.-S., A. J. Howard, and J. D. Crapo.** 1988. Nucleotide sequence of a rat glutathione peroxidase cDNA. Nucleic Acids Res. **16:**5207.
15. **Hubert, N., R. Walczak, P. Carbon, and A. Krol.** 1996. A protein binds the selenocysteine insertion element in the 3′-UTR of mammalian selenoprotein mRNAs. Nucleic Acids Res. **24:**464–469.
16. **Jaeger, J. A., D. H. Turner, and M. Zuker.** 1989. Improved predictions of secondary structure for RNA. Proc. Natl. Acad. Sci. USA **86:**7706–7710.
17. **Kim, I. Y., and T. C. Stadtman.** 1995. Selenophosphate synthetase: detection in extracts of rat tissues by immunoblot assay and partial purification of the enzyme from the archaean *Methanococcus vannielii*. Proc. Natl. Acad. Sci. USA **92:**7710–7713.
18. **Kollmus, H., L. Flohé, and J. E. G. McCarthy.** 1996. Analysis of eukaryotic mRNA structures directing cotranslational incorporation of selenocysteine. Nucleic Acids Res. **24:**1195–1201.
19. **Lee, B. J., P. J. Worland, J. N. Davis, T. C. Stadtman, and D. L. Hatfield.** 1989. Identification of a selenocysteyl-tRNA^{ser} in mammalian cells that recognizes the nonsense codon, UGA. J. Biol. Chem. **264:**9724–9727.

19a. **Leonard, J. L., D. M. Leonard, Q. Shen, A. P. Farwell, and P. E. Newburger.** 1996. Selenium-regulated translational control of heterologous gene expression: normal function of selenocysteine-substituted gene products. J. Cell. Biochem. **61:**410–419.

20. **Low, S. C., and M. J. Berry.** 1996. Knowing when not to stop: selenocysteine incorporation in eukaryotes. Trends Biochem. Sci. **21:**203–208.
21. **Low, S. C., J. W. Harney, and M. J. Berry.** 1995. Cloning and functional characterization of human selenophosphate synthetase, an essential component of selenoprotein synthesis. J. Biol. Chem. **270:**21659–21664.
22. **Martin, G. W., III, J. W. Harney, and M. J. Berry.** 1996. Selenocysteine incorporation in eukaryotes: insights into mechanism and efficiency from sequence, structure, and spacing proximity studies of the type 1 deiodinase SECIS element. RNA **2:**171–182.
23. **McCaughan, K. K., C. M. Brown, M. E. Dalphin, M. J. Berry, and W. P. Tate.** 1995. Translational termination efficiency in mammals is influenced by the base following the stop codon. Proc. Natl. Acad. Sci. USA **92:**5431–5435.
24. **Rocher, C., C. Faucheu, F. Hervé, C. Bénicourt, and J. L. Lalanne.** 1991. Cloning of murine SeGPx cDNA and synthesis of mutated GPx proteins in *E. coli*. Gene **98:**193–200.
25. **Roveri, A., A. Casasco, M. Maiorino, P. Dalan, A. Calligaro, and F. Ursini.** 1992. Phospholipid hydroperoxide glutathione peroxidase of rat testis. Gonadotropin dependence and immunocytochemical identification. J. Biol. Chem. **267:**6142–6146.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Shen, Q., F.-F. Chu, and P. E. Newburger.** 1993. Sequences in the 3′-untranslated region of the human cellular glutathione peroxidase gene are necessary and sufficient for selenocysteine incorporation at the UGA codon. J. Biol. Chem. **268:**11463–11469.
28. **Shen, Q., J. L. Leonard, and P. E. Newburger.** 1995. Structure and function of the selenium translation element in the 3′-untranslated region of human cellular glutathione peroxidase mRNA. RNA **1:**519–525.
29. **Shen, Q., P. A. McQuilkin, and P. E. Newburger.** 1995. RNA-binding proteins that specifically recognize the selenocysteine insertion sequence of human cellular glutathione peroxidase mRNA. J. Biol. Chem. **270:**30448–30452.
30. **Stadtman, T. C.** 1991. Biosynthesis and function of selenocysteine-containing enzymes. J. Biol. Chem. **266:**16257–16260.
31. **Thimmalapura, R. P., A. L. Burdsall, L. M. Oleksa, G. M. Chisolm, and D. M. Driscoll.** 1995. Rat phospholipid-hydroperoxide glutathione peroxidase: cDNA cloning and identification of multiple transcription and translation start sites. J. Biol. Chem. **270:**26993–26999.
32. **Walczak, R., E. Westhof, P. Carbon, and A. Krol.** 1996. A novel RNA structural motif in the selenocysteine insertion element of eukaryotic selenoprotein mRNAs. RNA **2:**367–379.
33. **Weitzel, F., F. Ursini, and A. Wendel.** 1990. Phospholipid hydroperoxide glutathione peroxidase in various mouse organs during selenium deficiency and repletion. Biochim. Biophys. Acta **1036:**88–94.
34. **Yamada, K.** 1995. A new translational elongation factor for selenocysteyl-tRNA in eucaryotes. FEBS Lett. **377:**313–317.
35. **Zinoni, F., A. Birkmann, W. Leinfelder, and A. Böck.** 1987. Cotranslational insertion of selenocysteine into formate dehydrogenase from *Escherichia coli* directed by a UGA codon. Proc. Natl. Acad. Sci. USA **84:**3156–3160.
36. **Zinoni, F., J. Heider, and A. Böck.** 1990. Features of the formate dehydrogenase mRNA necessary for decoding of the UGA codon as selenocysteine. Proc. Natl. Acad. Sci. USA **87:**4660–4664.