In mammalian selenoprotein mRNAs, the highly structured 3′ UTR contains selenocysteine insertion sequence (SECIS) elements that are required for the recognition of UGA as the selenocysteine codon. Our previous work demonstrated a tight correlation between codon-specific translational read-through and the activity of a 120-kDa RNA-binding protein that interacted specifically with the SECIS element in the phospholipid hydroperoxide glutathione peroxidase mRNA. This study reports the RNA binding and biochemical properties of this protein, SECIS-binding protein 2 (SBP2). We detected SBP2 binding activity in liver, hepatoma cell, and testis extracts from which SBP2 has been purified by anion exchange and RNA affinity chromatography. This scheme has allowed us to identify a 120-kDa polypeptide that co-elutes with SBP2 binding activity from wild-type but not mutant RNA affinity columns. A characterization of SBP2 biochemical properties reveals that SBP2 binding is sensitive to oxidation and the presence of heparin, rRNA, and poly(G). SBP2 activity elutes with a molecular mass of ~500 kDa during gel filtration chromatography, suggesting the existence of a large functional complex. Direct cross-linking and competition experiments demonstrate that the minimal phospholipid hydroperoxide glutathione peroxidase 3′ UTR binding site is between 82 and 102 nucleotides, which correlates with the minimal sequence necessary for translational read-through. SBP2 also interacts specifically with the minimally functional 3′ UTR of another selenoprotein mRNA, deiodinase 1.

The cotranslational insertion of selenocysteine (Sec) into a select group of both prokaryotic and eukaryotic proteins requires the presence of cellular factors and cis-acting sequences in their cognate mRNAs (reviewed in Refs. 1 and 2). In the case of bacteria, the structured cis-sequence (termed bacterial selenocysteine insertion sequence) lies immediately downstream from an in-frame opal codon (UGA) which directs the insertion of Sec (3). This process requires the activity of a specialized translation elongation factor (SELB) that interacts specifically with both the downstream sequences and with the selenocysteyl-tRNA Sec (4–7). The synthesis of selenocysteyl-tRNA requires the action of three other gene products: SELA, which converts seryl-tRNA Sec to selenocysteyl-tRNA Sec (8); SELC, which encodes the Sec RNA (9); and SELD, which synthesizes the selenocysteine donor selenophosphate (10).

The partially characterized process of Sec insertion into mammalian selenoproteins, although sharing some fundamental properties of the prokaryotic system, has many distinguishing features. First, the cis-sequences reside in the 3′ untranslated region (UTR) far downstream from the UGA codon (11). These sequences are predicted to form a stable stem-loop (see Fig. 1) and contain discrete, highly conserved selenocysteine insertion sequence (SECIS) elements that include the following: three consecutive, unpaired A residues in the terminal loop; AUGA in the stem 8–10 nucleotides (nt) 5′ of the terminal loop; and GA in the 3′ region of the stem across from the UGA element (11–13). Recent structural analyses have focused on the sequences in and around the UGA and GA SECIS elements that have been reported to form a non-Watson-Crick duplex (14, 15). The distance between the UGA codon and the SECIS elements is naturally variable between 500 and 5300 nt and therefore may represent an example of 3′-mediated translational regulation similar to that mediated by poly(A) and other regulatory sequences found in various 3′ UTRs (reviewed in Ref. 16).

Most selenoproteins are involved in redox reactions in which the active site Sec residue plays a central role in catalysis. This class of selenoproteins includes the bacterial formate dehydrogenases, the mammalian deiodinases, and glutathione peroxidases (GPxs), including phospholipid hydroperoxide glutathione peroxidase (PHGPx). Our studies are focused primarily on the synthesis of PHGPx, the enzymatic activity of which may play a role in the detoxification of pathogenic oxidized lipids (17). This enzyme is expressed in most tissues but is particularly abundant in testis, where it is targeted to the mitochondrial and nuclear membranes (18, 19). Outside the realm of oxidized lipids, the apparent association of a significant fraction of PHGPx with chromatin (20) is of particular interest in light of recent data that suggests that oxidized DNA may be a substrate for PHGPx enzymatic activity (21).

In an attempt to identify factors that may be involved in regulating Sec insertion, several groups have identified SECIS binding activities by both gel retardation and UV cross-linking studies. To date, a mammalian homolog of the bacterial selB gene has not been discovered, but direct binding studies using mammalian SECIS elements have identified factors that are potentially involved in linking the 3′ UTR to its potential targets such as ribosomes or tRNA. Shen et al. (22) have identified an RNA-protein complex that requires a perfectly base
Table I

| Mutant name | Oligo sequence |
|-------------|----------------|
| M129        | AGTACATTTTGCTGACCTTTCTGGA |
| M102        | ACACGCTGACCTTTCTGACCTTTGGA |
| M81         | AGTACATTTTGCAACCATAGCAGG |

* Nucleotides that differ from the wild-type are underlined.

**Materials and Methods**

**Synthetic RNAs**—The rat PHGPs 3′ UTR (nt 655–872) was cloned into the EcoRI/HindIII sites of pALTER-Ex1 (Promega). Deletion mutants (M129, M102, and M81) were constructed with synthetic oligonucleotides (listed in Table I) used to PCR amplify the terminal portions of the PHGPs 3′ UTR stem loop that then were cloned into the EcoRI and HindIII sites of pALTER-Ex1. Wild-type and mutant RNAs were synthesized with T7 RNA polymerase from HindIII linearized DNA templates. Deiodinase 1 constructs, a gift from Marla Berry (Harvard Medical School), were made as described (26) and were linearized with Dral. RNAs were radiolabeled by incorporation of [α-32P]UTP as described (27).

**Extract Preparation and Chromatography**—Fresh, pretrimmed rat testes were purchased from Pel-Freez (shipped on wet ice). The tissue was minced with a razor, and Buffer A (20 mM KPO4, pH 7.2, 100 mM KC1, 0.1 mM phenylmethylsulfonyl fluoride, 0.05% Tween-20, 5% glycerol, 2 mM DTT) was added to 2 ml/g of wet weight of tissue followed by centrifugation for 10 min at 18,000 × g. Pellets were resuspended in 1/10 of the original volume and either dialyzed or used directly for analysis or further purification. Protein concentrations were determined with the Bio-Rad protein assay normalized to bovine serum albumin standards.

For gel filtration chromatography, 0.5 mg of the ammonium sulfate fraction was applied to a 1 × 30-cm Superose-12 column (Amersham Pharmacia Biotech) equilibrated with Buffer A plus 1.1 M KCl. Proteins were eluted at a flow rate of 0.4 ml/min, and 1 ml fractions were collected. 100 μl of each fraction and 20 μl of the starting material were dialyzed against Buffer A in a multiwell microdialysis apparatus (Life Technologies, Inc.). Diazylated fractions (16 μl each) were analyzed for SBP2 activity by UV cross-linking. Sizing of SBP2 was performed by comparison to standards run under identical conditions: apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), cytochrome c (12.4 kDa).

**RNA Affinity Chromatography**—Wild-type and AUAG mutant PHGPs 3′ UTR RNAs were synthesized in bulk with Ribomax (Promega) transcription reagents and immobilized on CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) as described (28). The RNA beads (150–200 μg RNA/ml beads) were directly loaded into Amersham Pharmacia Biotech HR 0.5 × 5 cm FPLC columns which were subsequently equilibrated in Buffer A. Approximately 1 mg of protein from the ammonium sulfate fraction was loaded at 0.2 ml/min, and the column was washed in Buffer A plus 170 mM KCl until A280 returned to baseline. Proteins were eluted (0.5 ml/min) with a linear gradient (0.27–1.7 M KCl) in 10 min, followed by a step to 2 M KCl. Fractions (0.5 ml) were collected directly into 0.28 ml of saturated ammonium sulfate. After collection, fractions were incubated on ice for 30 min and spun at 18,000 × g in a 4 °C microcentrifuge for 10 min. The supernatant was removed and saved for later analysis. The pellets were resuspended in 50 μl of Buffer A and stored at −80 °C. The pellets were resuspended in 15 μl of 0.2 M NaOH, SDS sample buffer was added, and samples were electrophoresed on 8% SDS-polyacrylamide gels. The amount of activity derived from RNA affinity chromatography was calculated directly from analysis of limiting amounts of column fractions immediately after collection because the activity was not stable after this procedure even when stored at −80 °C. The yield of protein from RNA affinity chromatography was estimated by comparison to bovine serum albumin standards on a Coomassie-stained SDS-PAGE gel.

**UV Cross-linking Assay**—Extracts or column fractions were incubated with 20 fmol of [32P]-labeled (22,000 cpm/fmol) synthetic RNA for 30 min at 37 °C in a final volume of 20 μl containing Buffer A supplemented with 250 μg/ml Escherichia coli tRNA, 10 mM DTT, and 5 μg/ml soybean trypsin inhibitor (Sigma). Each reaction was treated with UV irradiation (Bio-Rad GS Genelinker) at 254 nm for 10 min in a 56-well tissue culture plate (Corning). The reaction was then treated with RNase A (1 mg/ml) for 30 min at 37 °C. Samples were analyzed by 8% SDS-PAGE and subjected to autoradiography. Quantitation was performed by PhosphorImager analysis (Molecular Dynamics). For specific activity determinations, data were obtained from a single phosphorimage scan except for the RNA affinity-derived activity, which was normalized to known quantities from the previous purification step. All determinations of specific activity and inhibitor effects were carried out under conditions of limiting protein within the linear range of the cross-linking assay as described in Tables II and III.

**Transient Transfections**—COS-7 cells were plated at 2 × 105 cells per 9.5-cm2 well in Dulbecco’s modified Eagle’s/Ham’s F-12 medium containing 10% fetal calf serum and 5 ng/ml Na2SeO3. At 70–80% confluency (18 h), 950 ng of test DNA was cotransfected with 50 ng of pRSV-β-galactosidase DNA using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s recommendations. At 48 h post-transfection, cells were harvested for detection of luciferase activity using the luciferase assay system (Promega) or β-galactosidase activity using LumiGal 530 assay reagent (Lumigen). Extracts were assayed using a ML2250 luminometer (Dynatech). Protein assays were performed using the Bio-Rad protein assay reagent. The luciferase activities were normalized to β-galactosidase activity, and the results were expressed as relative luminescence units/mg of protein.
Thickness 12

"apical"

AUGA SECIS Element

"basal"

FIG. 1. Proposed structure of the PHGPx 3’ UTR. Conserved SECIS elements are in boldface. Non-Watson-Crick base pairs are noted by the filled circles (●).

RESULTS

Tissue-specific Expression of SBP2—Our previous analysis of SBP2 binding activity was carried out with crude testicular extracts. These extracts contained several proteins identified by UV cross-linking that bound to the wild-type PHGPx 3’UTR, the most abundant of which migrated as a 55-kDa protein. Based on UV cross-linking and competition analysis, only a 120-kDa protein (SBP2) bound to the UTR in a sequence-specific manner (25). Mutational analysis of the AUGA and AAAA SECIS elements indicated that binding of SBP2 required the AUGA element (see diagram in Fig. 1). To support the idea that SBP2 is a general player in Sec insertion, and in order to identify the best tissue from which to obtain SBP2 activity, extracts derived from several rat tissues and McArdle 7777 cells using radiolabeled wild-type PHGPx 3’UTR or a mutant (AUGA → ACGA) previously shown to be unable to bind SBP2 (25). A 120-kDa cross-linking band that specifically binds the wild-type UTR was detectable only in testes, liver, and McArdle 7777 extracts. The lack of SBP2 cross-linking activity in kidney and spleen, as well as the reduced level observed in liver extracts, is potentially due to an inhibitor in these extracts because mixtures of these extracts with testicular extract inhibited binding (not shown). It is clear from this analysis that SBP2 functions in at least one other tissue. Among the extracts tested, that derived from testes had the highest specific activity. This result is consistent with the fact that the PHGPx mRNA and enzyme are over-represented in the testis relative to other tissues (19, 29, 30).

Characterization of SBP2—The apparent abundance of SBP2 in the testicular extracts assayed in Fig. 2 prompted our utilization of this tissue as a starting point for SBP2 purification. Although the UV cross-linking assay used to follow SBP2 binding activity is not highly quantitative, we estimate a 26-fold purification of activity after S-Sepharose chromatography and ammonium sulfate precipitation (Table II). As expected, SBP2 may have the characteristics of a basic protein or complex as evidenced by its preferential interaction with cation exchange matrices, such as S-Sepharose, at neutral pH. SBP2 does bind to a MonoQ column (anion exchange) when the pH was raised to 8.0, but significant losses of activity undermined its utility in the purification scheme. SBP2 activity eluted in a single peak from the S-Sepharose column at a point in the gradient corresponding to ~200 mM KCl. The activity was subsequently concentrated, and the excess KCl removed by 35% ammonium sulfate precipitation. The mixture obtained at this step was used for the analyses described below and is referred to as partially purified SBP2. This purification scheme was sufficient to remove the major contaminating cross-linking proteins detected in S100 extracts, most notably the abundant 55-kDa protein (compare crude testis extracts to the ammonium sulfate fraction in Fig. 2). The lower molecular weight bands detected by UV cross-linking in the ammonium sulfate fraction do not appear consistently and may represent variations in the recovery of proteins that bind to the double stranded stem of the full-length PHGPx 3’UTR (compare Figs. 2 and 6). The specificity of partially purified SBP2 binding was verified by virtue of its ability to cross-link to wild-type but not mutant (AUGA → ACGA) PHGPx 3’UTRs (Fig. 2).

The conditions under which maximal UV cross-linking activity can be detected for partially purified SBP2 were analyzed. The optimal reaction conditions include 50–100 mM KCl, pH 6.5–7.0, at 30–37 °C. The addition of magnesium did not augment cross-linking, and binding was not inhibited by up to 5 mM EDTA, indicating that a metal co-factor is not necessary for this interaction. SBP2 binding was not affected by repeated freeze/thaw cycles and was cryostable. Nonionic detergents had no deleterious effect on binding, and SBP2 binding was restored after treatment with 2 M guanidine HCl followed by dialysis. Denaturation, however, in SDS or 6 M guanidine was irreversibly inhibitory to binding activity. The effects of a variety of compounds on the level of SBP2 UV cross-linking found in partially purified preparations are summarized in Table III. We chose to analyze an array of nucleic acids that may compete...
for binding, as well as several divalent cations that may facilitate the binding activity. In addition, we tested the effect of sodium selenite to determine whether binding activity is regulated by selenium concentration. Two-fold serial dilutions of each effector were assayed in standard cross-linking reactions with limiting amounts of partially purified SBP2 (2 μg), and the concentration at 50% inhibition (IC50) was determined by PhosphorImager-based quantitation. Table III lists the effect of various DNA and RNA binding proteins on the binding activity of SBP2. As noted by arrows.

**Analysis of potential effectors of SBP2 binding activity**

2-fold serial dilutions of the substances listed below were added to standard cross-linking reactions containing limiting amounts of partially purified SBP2 (2 μg), and the concentration at 50% inhibition (IC50) was determined by PhosphorImager-based quantitation.

| Substance | IC50 | Weak negative effect | IC50 | Strong negative effect | IC50 |
|-----------|------|----------------------|------|-----------------------|------|
| Na2SeO3  | >100 μM | Na2SeO3 | 72 μM | rRNA | 1 mg/ml |
| Poly(A)  | >25 μg/ml | MgCl2 | 3.8 mM | NiCl2 | 0.2 mM |
| Poly(C)  | >25 μg/ml | MnCl2 | 2.3 mM | Poly(G) | 3 μg/ml |
| Poly(U)  | >25 μg/ml | CaCl2 | 3.9 mM | Heparin | <0.5 mg/ml |
| pI:πC   | >50 μg/ml |  |  |  |  |
| DNAa     |  >50 μg/ml |  |  |  |  |
| EDTAc    |  >5 mM |  |  |  |  |

a E. coli 16 S and 23 S rRNA.

b Salmon sperm DNA.

c RNA affinity chromatography. Because of low yield, the amount of total protein in this fraction was estimated by comparison to standards in a Coomassie Blue-stained SDS gel.

In this case, the majority of SBP2 activity eluted in a major peak corresponding to ~500 kDa and a minor peak corresponding to ~200 kDa. The recovery of SBP2 activity under these conditions was good (~70%), thereby discounting the possibility that we were only observing a small fraction of the input material. These results suggest that the 120-kDa SBP2 as detected by UV cross-linking is part of a homogeneous or heterogeneous multiprotein complex that can be only partially dissociated under high ionic strength conditions.

**SBP2 Binding Is Redox-sensitive**—As it has been shown that many DNA and RNA-binding proteins require free cysteine residues for binding activity (31, 32), we studied SBP2 binding activity in the context of altered redox potential. Extracts made in buffer lacking DTT showed little if any SBP2 binding activity, whereas the addition of 10–300 mM DTT to the reaction stimulated SBP2 cross-linking activity. Furthermore, pretreatment of crude or partially purified sources of SBP2 with the oxidizing agent diamide eliminated binding activity, and this inhibition was reversed when 0.1 M DTT was added after diamide treatment (not shown). These results suggest a role for free cysteine residues in the SBP2 protein. To further this analysis, extracts were pretreated with the irreversible sulfhydryl modifying agent N-ethylmaleimide. Pretreatment with 5 mM N-ethylmaleimide completely eliminated binding activity even after the addition of excess DTT (not shown). Together, these results strongly suggest that free cysteine residues are in some way required for RNA binding.

**Identification of SBP2 Polypeptide**—Further purification of SBP2 by means of RNA affinity chromatography has allowed us to achieve a 1500-fold purification (Table II) and identify a candidate polypeptide corresponding to SBP2. Partially purified SBP2 was applied to RNA affinity columns composed of wild-type or the AUGA deletion mutant (ΔAUGA) PHGPx 3′ UTR RNA covalently attached to CNBr-activated Sepharose 4B. Bound proteins were eluted with a biionic gradient from 0.1–2.0 M KCl. UV cross-linking analysis demonstrates that SBP2 elutes from the wild-type column under high salt conditions, suggesting a high affinity electrostatic interaction (Fig. 4B). In the case of the mutant column, SBP2 activity was detected both in the flow-through fractions as well as fractions eluted early in the gradient (Fig. 4A). As indicated by the chromatogram in Fig. 4C, this procedure separated SBP2 activity from the bulk of the proteins that bind the wild-type RNA column, which eluted earlier in the gradient at ~300–400 mM KCl. By raising the KCl concentration to 0.27 M for the initial binding conditions, a significant enhancement of purification was obtained, as revealed by SDS-PAGE analysis of peak fractions from wild-type and mutant RNA affinity chromatography (Fig. 5). Under these conditions, a 120-kDa polypeptide was clearly selected on the wild-type but not the mutant RNA column. The specificity of SBP2 derived from RNA affinity chromatography was verified by its inability to cross-link to the
says to wild-type 32P-labeled PHGPx 3 M81, 81 nt) were used as competitors in UV-cross-linking as-
gressively shorter UTRs lacking sequences constituting the
putative basal stem but retaining the three conserved SECIS
elements (Fig. 6). Mutant UTRs (M129, 129 nt; M102, 102 nt; M81, 81 nt) were used as competitors in UV-cross-linking as-
says to wild-type 32P-labeled PHGPx 3’ UTR. As shown in Fig. 6,B, only the shortest mutant (M81) was unable to compete
effectively. The concentration of cold RNA necessary for a 50% reduction in cross-linking to the wild-type probe is shown for quantitative comparison (Table IV). M129 and M102 competed as efficiently as the wild-type UTR, whereas M81 competed least effectively, approximately 25 times less well than the other constructs. Interestingly, the proposed structure of the M81 construct lacks a significant number of paired residues basal to the AUGA SECIS element, suggesting that a paired stem may stabilize the apical structures.

To assay functionality, these same constructs were cloned into the 3’ UTR of a modified luciferase mRNA which contains an in-frame UGA codon, a system used extensively in our previous study to establish the relationship between SBP2 binding and translational read-through (25). The ability of these mutant UTRs to allow read-through of the in-frame UGA was determined by transfection of the luciferase constructs into COS-7 cells, which were subsequently extracted and assayed for luciferase activity. When compared with a wild-type control, the shorter basal stem constructs (M129 and M102) possessed lower luciferase activity, whereas the smallest construct (M81) resulted in no read-through above background (Table IV). These changes in translatability cannot be accounted for by changes in mRNA levels as determined by Northern analysis of the mutant RNAs (not shown). Taken together, the RNA binding and luciferase read-through data indicate that the minimally functional PHGPx 3’ UTR is between 82 and 102 nt.

Although it is clear from our results that SBP2 requires the AUGA SECIS element and is likely binding to that site, it is possible that SBP2 also makes contacts in the 5’ UTR or coding region of the PHGPx mRNA in its potential role of linking the 3’ UTR with translational machinery. Even though the PHGPx 3’ UTR is sufficient to allow read-through of a UGA in a heterologous message (luciferase), it is possible that SBP2 works with a higher efficiency in the presence of coding region or 5’ binding targets. To address this possibility, we analyzed the ability of the PHGPx coding region to compete for binding to the PHGPx 3’ UTR. Full-length PHGPx mRNAs containing either wild-type or mutant 3’ UTRs were used as competitors in cross-linking reactions with partially purified SBP2 (see below) and the 32P-labeled wild-type 3’ UTR. Although the wild-type full-length RNA competes as effectively as the 3’ UTR alone (IC50 = 3-fold), more than 100-fold excess of mutant RNA is required to achieve the same effect.

**SBP2 Binds to the Deiodinase 1 3’ UTR**—Having previously established that SBP2 binds to both the full-length PHGPx and GPx 3’ UTRs (25), we desired to determine whether or not the SBP2 binding site might be present in another class of selenoprotein mRNAs. To this end, we analyzed by UV cross-linking and competition experiments the ability of SBP2 to interact specifically with the 3’ UTR of the selenoprotein deiodinase 1 (D1) mRNA. A comparison of the PHGPx 3’ UTR sequence and that found in D1 is shown in Fig. 7A. Although the conservation of sequences making up the SECIS elements is clear, similarities outside those regions are quite limited. We therefore predict that if SBP2 does interact with the D1 3’ UTR, then it is likely to be making contact with a SECIS element, specifically AUGA.

Fig. 7B shows UV cross-linking of partially purified SBP2 to the minimally functional rat D1 3’ UTR, which is 42 nt in length. SBP2 did not cross-link to a D1 3’ UTR with a SECIS mutation (AUGA → AGA) that was previously shown to abolish Sec incorporation (33). For comparison, cross-linking to the full-length 204 nt PHGPx 3’ UTR is also shown. Interestingly, the lower molecular weight bands detected by the PHGPx probe are diminished in intensity with the D1 probe, which lacked the basal stem. These proteins are likely binding to the long stem of the PGHPx 3’UTR, as they were also detected...
when a version of the D1 3' UTR with a longer stem is used (not shown). Fig. 7C shows competition experiments that tested the ability of unlabeled RNAs corresponding to the D1 constructs to effectively compete for binding to the labeled PHGPx 3' UTR. The concentrations of competitor necessary to inhibit 50% of the binding activity are shown for quantitative comparison. The D1 3' UTR competes 50% of the signal at a 14-fold molar excess, whereas the wild-type PHGPx 3' UTR competes 50% of the signal at less than a 1.5-fold excess. From this analysis, it is clear that SBP2 is not solely associated with the anti-oxidative class of selenoproteins, and it is likely to be in direct contact with the AUGA SECIS element within the selenoprotein MRNAs. Consistent with similar experiments with the PHGPx 3' UTR (see below), the basal stem does not seem to be necessary for SBP2 binding, but there does appear to be some enhancement when a portion of the base paired structure is present.

**DISCUSSION**

Here we report the characterization and purification of a SECIS-binding protein (SBP2) derived from rat testicular extracts. This study represents the first biochemical character-
TABLE IV
Translational read-through and competition for binding directed by deletion mutants of the rat PHGPx 3' UTR

| 3' UTR | IC_{50} (fold molar excess) | Read-through |
|--------|-----------------------------|--------------|
| Wild-type | <1.5 | 100 |
| M129 | 2 | 58.2 (±9.2) |
| M102 | 2 | 26.6 (±0.6) |
| M81 | 52 | 6.3 (±1.3) |
| None | ND | 5.6 (±0.7) |

* Not determined.

a g

FIG. 7. SBP2 binding to the D1 3' UTR. A, sequence comparison of the terminal portions of the rat D1 and rat PHGPx 3' UTRs. Identical bases are in boldface, and SECIS elements are underlined. The minimally functional D1 construct of 42 nt is denoted by the arrows. B, 2 μg of partially purified SBP2 was incubated with 32P-labeled full-length PHGPx 3' UTRs. Identical bases are in boldface, and SECIS elements are underlined. The minimally functional D1 construct of 42 nt is denoted by the arrows. C, 1.5-50-fold molar excess of various competitor RNAs (as indicated to the left of each panel) were incubated with 32P-labeled wild-type PHGPx 3' UTR in the UV cross-linking assay.

SBP2. Another SBP in the 60 kDa range has also been identified (34), but an investigation of binding specificity has not been reported.

We have purified SBP2 to a point at which a clear identification of a corresponding 120-kDa polypeptide was possible based on selective binding to a wild-type RNA affinity matrix. It is clear from Fig. 5 that SBP2 is not the only protein that can be isolated by RNA affinity chromatography. Indeed, the analysis of the major factors in this preparation may quickly identify other factors interacting with distinct regions of the 3' UTR. SBP2 appears to be a basic protein that preferentially interacts with structured RNA molecules as evidenced by the inability of nonstructured ribohomopolymers, DNA and poly(I): poly(C), to compete for binding. It does not appear to require any co-factors and is stable once extracted from cells or tissues. The biochemical properties of SBP2 are consistent with its potential role in coordinating the interaction between highly structured RNA (the 3' UTR) and other components of the Sec insertion machinery.

Of particular interest is the relatively large aggregate molecular weight of SBP2 activity as determined by gel filtration chromatography, which suggests the specific association of homologous or heterologous factors. The isolation of SBP2 by RNA affinity chromatography should provide the means (i.e. anti-SBP2 antibodies and/or SBP2 column matrix) with which to identify the associated factors and subsequently determine their biological activities. It seems likely, based on this observation, that the role of RNA-binding proteins in Sec insertion will exceed in complexity that found in prokaryotes, in which only a specialized elongation factor (SELB) is necessary. According to the data presented in this work, SBP2 does not appear to be involved in binding to other elements within the PHGPx mRNA, but it remains a possibility that other factors may mediate protein/protein or RNA/RNA contacts at the UGA codon.

Another significant finding from this study is that SBP2 binding activity is not restricted to the testis. Extracts from liver and a hepatoma cell line are in possession of an apparently identical binding activity, and it is quite possible that other tissues do as well, even though cross-linking activity could not be detected in kidney or spleen extracts. The inhibition of SBP2 binding activity in the presence of kidney, liver, and spleen extracts indicates the existence of a potentially specific inhibitor. Work is in progress to determine whether or not this inhibitor is a discrete and specific entity. Together with the fact that SBP2 is known to bind the PHGPx, GPx (25) and D1 (this report) 3' UTRs, these data support the idea that SBP2 is a general factor involved in Sec incorporation and is not specific to testicular expression of PHGPx.

In this report, we also set out to establish SBP2 as a general factor involved in Sec insertion by virtue of its ability to bind the deiodinase 1 3' UTR. In fact, this experiment indicates that SBP2 binds to the D1 3' UTR with the same sequence specificity with which it binds to PHGPx mRNA in that the UAGA SECIS element is required in both cases. For PHGPx, we analyzed mutant RNA molecules that lacked varying amounts of the putative basal stem region and found that only the construct that lacked a significant amount of paired sequences basal to the SECIS elements was unable to bind. In addition, the PHGPx 3' UTRs with shortened stems were not as efficient at directing read-through in our luciferase assay, indicating compromised functionality as well. It is clear from this study that the basal stem of the PHGPx 3' UTR does not play a significant role in SBP2 binding, but that a small amount of

2 P. R. Copeland and D. M. Driscoll, unpublished results.
this structure is required for optimal binding and functionality as determined by the read-through assay. In contrast, Martin et al. (26) found that a minimal PHGPx 3’ UTR that lacked any sequences basal to the UGA SECIS element was sufficient when placed downstream of the deiodinase coding region (26). This study did not use full-length or other PHGPx constructs for comparison, however, and it is possible that differences in the assay systems may explain the apparent discrepancy. From our study, we must conclude that the sequence required for the assay systems may explain the apparent discrepancy. From this structure is required for optimal binding and functionality as determined by the read-through assay. In contrast, Martin et al. (26) found that a minimal PHGPx 3’ UTR that lacked any sequences basal to the UGA SECIS element was sufficient when placed downstream of the deiodinase coding region (26). This study did not use full-length or other PHGPx constructs for comparison, however, and it is possible that differences in the assay systems may explain the apparent discrepancy. From our study, we must conclude that the sequence required for the assay systems may explain the apparent discrepancy. From

The possibility that SBP2 functions to link the 3’ UTR with the UGA codon itself prompted our investigation of SBP2 binding sites in the 5’ UTR and coding region of the PHGPx mRNA. Competition studies using full-length PHGPx mRNAs with either wild-type or mutant 3’ UTRs demonstrated that there are no high affinity binding sites other than at the UGA SECIS element. It is possible, however, that SBP2 does have other RNA targets, such as the Sec tRNA (as in the case of SELB, the bacterial SECIS-binding protein) or ribosomal RNA. Based on these binding studies, therefore, we propose that SBP2 directly interacts with the UGA SECIS element.

Based on the results in this study, we envision that SBP2 plays a central role as a member of a heterogeneous or homogeneous complex that binds to selenoprotein mRNAs at the 3’ UTR and perhaps elsewhere in the message, providing the ribosome the information necessary to bypass termination and incorporate selenocysteine. This model does not necessitate a direct interaction between SBFs and the Sec tRNA, as it is possible that they may only be involved in preventing termination long enough for the limited supply of Sec tRNA to gain access.

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REFERENCES
1. Stadtmann, T. C. (1996) Annu. Rev. Biochem. 65, 83–100
2. Low, S. C., and Berry, M. J. (1996) Science 273, 1966–1968
3. Huttenhofer, A., Westhof, E., and Bock, A. (1996) RNA 2, 354–366
4. Baron, C., Heider, J., and Bock, A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4181–4185
5. Chen, G. F., Fang, L., and Inouye, M. (1993) J. Biol. Chem. 268, 23128–23131
6. Forchhammer, K., and Bock, A. (1991) J. Biol. Chem. 266, 6324–6328
7. Zinoni, P., Heider, J., and Bock, A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4660–4664
8. Heider, J., Baron, C., and Bock, A. (1992) EMBO J. 11, 3759–3766
9. Martin, G. W., Harney, J. W., and Berry, M. J. (1998) Nature 333, 723–725
10. Ehrenreich, A., Forchhammer, K., Tormay, P., Veprek, B., and Bock, A. (1992) Eur. J. Biochem. 206, 767–773
11. Berry, M. J., Banu, L., Chen, Y. Y., Mandel, S. J., Kieffer, J. D., Harney, J. W., and Larsen, P. R. (1991) Nature 353, 273–276
12. Berry, M. J., Banu, L., Harney, J. W., and Larsen, P. R. (1993) EMBO J. 12, 3315–3322
13. Shen, Q., Chu, F. F., and Newburger, P. E. (1993) J. Biol. Chem. 268, 11463–11469
14. Walczak, R., Westhof, E., Carbon, P., and Krol, A. (1996) RNA 2, 367–379
15. Walczak, R., Carbon, P., and Krol, A. (1998) RNA 4, 74–84
16. Sachs, A. B., Sarnow, P., and Hentze, M. W. (1997) Cell 89, 831–838
17. Thomas, J. P., Maiorino, M., Ursini, F., and Girotti, A. W. (1990) J. Biol. Chem. 265, 454–461
18. Godeas, C., Sandri, G., and Panfili, E. (1994) Biochim. Biophys. Acta 1191, 147–150
19. Pushpa-Rekha, T. R., Burdsall, A. L., Oleksa, L. M., Chisolm, G. M., and Driscoll, D. M. (1995) J. Biol. Chem. 270, 26993–26999
20. Godeas, C., Tramer, F., Micali, F., Roveri, A., Maiorino, M., Nissi, C., Sandri, G., and Panfili, E. (1996) Biochem. Mol. Med. 50, 118–124
21. Bao, Y., Jenth, P., Manners, B., and Williamson, G. (1997) FEBS Lett. 410, 210–212
22. Shen, Q., McQuilkin, P. A., and Newburger, P. E. (1995) J. Biol. Chem. 270, 30448–30452
23. Shen, Q., Leonard, J. L., and Newburger, P. E. (1996) RNA 2, 519–525
24. Shen, Q., Wu, H., Leonard, J. L., and Newburger, P. E. (1998) J. Biol. Chem. 273, 6440–6446
25. Leson, A., Mehta, A., Singh, R., Chisolm, G. M., and Driscoll, D. M. (1997) Mol. Cell. Biol. 17, 1977–1985
26. Martin, G. W., Harney, J. W., and Berry, M. J. (1998) RNA 4, 65–73
27. Driscoll, D. M., Lakhe-Reddy, S., Oleksa, L. M., and Martinez, D. (1993) Mol. Cell. Biol. 13, 7288–7294
28. Kaminiski, A., Hunt, S. L., Patton, J. G., and Jackson, R. J. (1995) RNA 1, 924–928
29. Weitzel, F., Ursini, F., and Wendel, A. (1990) Biochim. Biophys. Acta 1036, 88–94
30. Zhang, L. P., Maiorino, M., Roveri, A., and Ursini, F. (1989) Biochim. Biophys. Acta 1006, 140–143
31. Bandopadhayay, S., and Gronostajski, R. M. (1994) J. Biol. Chem. 269, 29949–29955
32. Hentze, M. W., Rouault, T. A., Harford, J. B., and Klausner, R. D. (1989) Science 244, 357–359
33. Martin, G. W., Harney, J. W., and Berry, M. J. (1996) RNA 2, 171–182
34. Hubert, N., Walczak, R., Carbon, P., and Krol, A. (1996) Nucleic Acids Res. 24, 464–469