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# A novel RNA binding protein, SBP2, is required for the translation of mammalian selenoprotein mRNAs

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**In eukaryotes, the decoding of the UGA codon as selenocysteine (Sec) requires a Sec insertion sequence (SECIS) element in the 3′ untranslated region of the mRNA. We purified a SECIS binding protein, SBP2, and obtained a cDNA clone that encodes this activity. SBP2 is a novel protein containing a putative RNA binding domain found in ribosomal proteins and a yeast suppressor of translation termination. By UV cross-linking and immunoprecipitation, we show that SBP2 specifically binds selenoprotein mRNAs both *in vitro* and *in vivo*. Using $^{75}$Se-labeled Sec-tRNA$^{Sec}$, we developed an *in vitro* system for analyzing Sec incorporation in which the translation of a selenoprotein mRNA was both SBP2 and SECIS element dependent. Immunodepletion of SBP2 from the lysates abolished Sec insertion, which was restored when recombinant SBP2 was added to the reaction. These results establish that SBP2 is essential for the co-translational insertion of Sec into selenoproteins. We hypothesize that the binding activity of SBP2 may be involved in preventing termination at the UGA/Sec codon.**

*Keywords*: RNA binding protein/SECIS element/selenocysteine/translation/3′ UTR

## Introduction

The incorporation of selenocysteine (Sec) into a select group of both prokaryotic and eukaryotic proteins creates a unique class of enzymes. Most selenoproteins are involved in redox reactions in which the active site Sec residue plays a central role in catalysis (reviewed in Low and Berry, 1996; Stadtman, 1996). This class of selenoproteins includes the bacterial formate dehydrogenases, the mammalian deiodinases, thioredoxin reductases, and glutathione peroxidases (GPx) including phospholipid hydroperoxide glutathione peroxidase (PHGPx). Our studies are focused primarily on the synthesis of PHGPx, whose enzymatic activity may play a protective role in the development of atherosclerotic lesions by detoxifying pathogenic lipids (Thomas *et al*., 1990). An important non-enzymatic function for PHGPx was recently described by Ursini *et al*. (1999), who showed that PHGPx acts as an oxidatively cross-linked structural protein in the midpiece of mature spermatozoa. That any single selenoprotein is essential for life remains to be determined, but their combined loss by means of disruption of the Sec-tRNA$^{Sec}$ gene in the mouse results in early embryonic lethality (Bosl *et al*., 1997).

Sec incorporation embodies a significant exception to the interpretation of the universal genetic code as it requires the recoding of a UGA stop codon when it is found in the context of a specific, downstream RNA structure. In the case of bacteria, the structured *cis*-sequence (termed bacterial Sec insertion sequence, SECIS) lies immediately downstream from an in-frame opal codon (UGA) that directs the insertion of Sec (Huttenhofer *et al*., 1996). 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$^{Sec}$ (Sec-tRNA$^{Sec}$) (Zinoni *et al*., 1990; Heider *et al*., 1992; Baron *et al*., 1993; Chen *et al*., 1993). The synthesis of Sec-tRNA$^{Sec}$ requires the action of three other gene products: SELA, which converts Ser-tRNA$^{Sec}$ to Sec-tRNA$^{Sec}$ (Forchhammer and Bock, 1991); SELC, which encodes tRNA$^{Sec}$ (Leinfelder *et al*., 1988); and SELD, which synthesizes the selenium donor selenophosphate (Ehrenreich *et al*., 1992).

The process of Sec incorporation into mammalian selenoproteins remains in the initial phases of discovery. While sharing some fundamental properties of the prokaryotic system, it has many distinguishing features. The required *cis*-sequences reside in the 3′ untranslated region (UTR) far downstream from the UGA codon (Berry *et al*., 1991a). The distance between the UGA codon and this element is naturally variable between 500 and 5300 nucleotides (nt). These sequences are predicted to form a stable stem–loop commonly referred to as a SECIS. Within the SECIS element are several short, discrete, highly conserved sequences, which include three consecutive, unpaired A residues in the terminal loop, AUGA in the stem 8–10 nt 5′ of the terminal loop, and GA in the 3′ region of the stem across from the AUGA element (Berry *et al*., 1991a, 1993; Shen *et al*., 1993). Recent structural analyses have focused on the sequences in and around the AUGA and GA elements, which have been reported to form a non-Watson–Crick duplex (Walczak *et al*., 1996, 1998). These structures appear to fall into two distinct classes depending on whether the terminal region is comprised of a single loop (form 1) or an adenosine bulge and smaller terminal loop (form 2; Grundner-Culemann *et al*., 1999).

In the absence of a mammalian homolog of the bacterial *selB* gene, efforts have focused on the identification of proteins that bind specifically to the SECIS element as it is likely that such a factor would be involved in linking

the SECIS element with the Sec-tRNA$^{Sec}$ or with a selenoprotein-specific elongation factor. Several groups have identified SECIS binding activities (SBPs) by both gel retardation and UV cross-linking studies, but a relationship between these factors and Sec insertion has not been established (Haas and Velten, 1992; Shen *et al*., 1993, 1995; Hubert *et al*., 1996). One potential reason for the lack of functional data for SBPs is the lack of a reliable *in vitro* assay for Sec incorporation.

Making use of the fact that PHGPx is highly expressed in testis, we previously identified a 120 kDa SECIS binding protein (SBP2) that interacted specifically with both PHGPx and GPx 3′ UTRs (Lesoon *et al*., 1997). SBP2 activity was tightly correlated with selenoprotein synthesis since SBP2 binding and translational readthrough of the UGA codon were both eliminated or greatly reduced by mutations to the AUGA element. Recently, we reported the biochemical and RNA binding characteristics of SBP2, which was purified from rat testicular extracts (Copeland and Driscoll, 1999). Here, we utilized the same purification scheme and obtained sufficient SBP2 protein for peptide sequencing and subsequent cloning of the SBP2 cDNA. SBP2 is a novel protein of 846 amino acids that contains a putative RNA binding domain similar to that found in the yeast SUP1 omnipotent suppressor of translation termination (Koonin *et al*., 1994). We also report the first definitive incorporation of Sec into a selenoprotein translated in reticulocyte lysates. Using antibodies raised against bacterially expressed SBP2, we show that immunodepletion of SBP2 results in a complete loss of Sec incorporation. By adding back *in vitro* translated SBP2 to the depleted translation extracts, Sec incorporation was restored. These results demonstrate that SBP2 is required for Sec insertion and that this protein is limiting in reticulocyte lysates.

## Results

### *Cloning of SBP2 cDNA*

We previously reported the purification of SBP2 by RNA affinity chromatography and the identification of a 120 kDa polypeptide that co-fractionated with SBP2 binding activity (Copeland and Driscoll, 1999). Using ~50 g of trimmed rat testes, SBP2 polypeptide was pooled from several rounds of purification and separated from minor contaminants by SDS–PAGE. The band corresponding to SBP2 was excised and subjected to trypsin digestion, and the peptides were sequenced by tandem electrospray mass spectrometry (MS). A total of 14 peptides were sequenced by tandem liquid chromatography (LC)/MS, four of which were of sufficient length and quality to be used as a query of the DDBJ/EMBL/GenBank databases using the tBLASTn program. While no matches were found in the non-redundant database, several rat, mouse and human expressed sequence tags (ESTs) were identified. One of the rat EST sequences (DDBJ/EMBL/GenBank accession No. H31811) was obtained from the American Type Culture Collection (ATCC), sequenced and found to contain an open reading frame (ORF) that encoded four other SBP2 peptides identified by LC/MS. The ORF contained in this EST (1413 bp) was not of sufficient length to encode a 120 kDa protein. Using the 5′ region of this DNA, primers were designed for 5′ rapid amplification of cDNA ends (RACE) from a rat testis λgt11 cDNA library. Two RACE clones contained overlapping sequence with EST H31811 and were analyzed further. The longer of the clones (2.2 kb) contained the remainder of the SBP2 coding sequence, and a full-length cDNA clone was constructed as described in Materials and methods.

The deduced SBP2 amino acid sequence is shown in Figure 1A. The complete cDNA encodes a protein of 846 amino acids with a predicted mol. wt of 93.3 kDa. The discrepancy between the predicted molecular weight and the molecular weight observed by SDS–PAGE (120 kDa) is apparently due to aberrant migration during electrophoresis as *in vitro* translation of the coding region alone in reticulocyte lysates yields a major product migrating at 120 kDa (data not shown). While SBP2 is not homologous to any known proteins, the C-terminal 429 amino acids of SBP2 bear 46% identity with a protein of unknown function (human hypothetical protein KIAA0256, DDBJ/EMBL/GenBank accession No. D87445). The sequence alignment of SBP2 and KIAA0256 is shown in Figure 1B. The most notable feature of this protein is a putative RNA binding domain, which is conserved among several ribosomal proteins as well as the SUP1 protein, a translation termination supressor in yeast (Koonin *et al*., 1994). Another striking feature of the SBP2 sequence is a lysine-rich region from amino acids 368–382, which is predicted to be a strong nuclear localization signal.

### *Expression of SBP2*

To analyze the expression pattern of SBP2 mRNA, a Northern blot derived from poly(A)$^+$ RNA from various rat tissues was probed with the 3′ half of the SBP2 coding region (Figure 2). Two larger transcripts (4 and 3.5 kb) are detectable in all tissues tested, while a smaller (2.5 kb) transcript, which is of considerable abundance relative to the larger transcripts, is only observed in testis. The SBP2 clone used in this study generates a 3173 nt transcript (2541 nt of coding sequence plus a 632 nt 3′ UTR truncated at the polyadenylation site). Thus, the smaller transcript present in testis may correspond to a splice variant lacking the 3′ UTR or some of the coding region. The origin of the various transcripts is currently under investigation. The abundance of the smaller transcript in testis is coincident with the overexpression of SBP2 protein in this tissue, as determined by immunoprecipitation and Western blotting (data not shown), suggesting that the smaller transcript may be responsible for this high level of expression.

### *Binding properties of recombinant SBP2*

To verify that the sequence cloned encodes a sequence-specific RNA binding protein, we tagged both full-length SBP2 and a C-terminal portion (amino acids 280–846) with an 8-amino-acid Strep tag. These proteins were overexpressed in bacteria and purified by affinity chromatography. While the C-terminal protein was expressed efficiently, very little stable full-length protein was obtained. In a UV cross-linking experiment with radiolabeled PHGPx 3′ UTRs, both of the recombinant SBP2 proteins bound specifically to the wild-type but not to the AUGA→ACGA mutant PHGPx 3′ UTRs (Figure 3A). This mutation is in one of the conserved motifs within the SECIS element and has previously been shown to

**A**

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1   MASERPREPE GEDSIKLSAD VKPFVPKFAG LNVAWSESSE ACVFPGCAAT YYPFVQESPA
61  AEQKMYPEDM AFGAPAFPAQ YVSSEIALHP FAYPTYALES TQSVCSVPTL QYDYSQAQCH
121 PGFRPAKPRN EHACPPQEAK CVFKKKSSDE RRAWEEQKSS NRRADGAVPC EARPARGSCH
181 LKSDGYHKRP DRKSRILTKS ASTSKPEFEF SRLDFPELQS PKNSNLPETQ KQPRWGPLGP
241 AASNMSLLGE AGKPVADMVE GKMVKTDHTD GAVTNNAATS SPSCTRELSW TPMGYIVRQT
301 VSSDSAAATE TVNSIINLKK TTSSADAKNV SVTSEALSSD PSFSREKRVH PGPKAKASQG
361 SELEQNESSK KNKKKKEKSK SSYEVLPVQE PPRIEDAEEF PNLSVASERR HRGESPKLQS
421 KQQAQNDFKT GGKKSQVPVQ LDLGGMLAAL EKQQHAPHAK PSSRPVVFSV GAVPVLSKDA
481 SSGERGRRSS QVKTPHNPLD SSAPLMKKGK QREIPKAKKP TSLKKIILKE RQERMQQRLQ
541 ESAVSPTVAS DDSQDVESGV TNQIPSPDNP TGPEKTEEPM SSTPVVEGES EEPAGTEFQR
601 DPEACQPAPD SATFPKIHSR RFRDYCSQML SKEVDACVTG LLKELVRFQD RMYQKDPVKA
661 KTKRRLVLGL REVLKHLKLR KLKCIIISPN CEKTQSKGGL DDTLHTIIDC ACEQNIPFVF
721 ALNRKALGRS LNKAVPVSIV GIFSYDGAQD QFHKMVELTM AARQAYKTML KTMRQEQAGE
781 PGPQTPPSPP MQDPIQSTDE GTLASTGEEP HYIEIWRKHL EAYSQHALEL EDSLEASTSQ
841 MMNLNL
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**B**

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SBP2 : 418 LQSKQQAQNDFKTGGKKSQVPVQLDLGGMLAALEKQQHAPHAKP--SSRPVVFSV-GAVP 474
           ++ K+  +   K  GKK++ PVQLDLG MLAALEKQQ A  A+   ++RP+ ++V  A
HHP  : 1   MEQKKLQEALSKAAGKKNKTPVQLDLGDMLAALEKQQQAMKARQITNTRPLSYTVVTAAS 60

SBP2 : 475 VLSKDASSGERGRRSSQVKTPHNPLDSSAPLMKKGKQREIPKAKKPTSLKKIILKERQER 534
             +KD+++ +   +S    T  N +D ++   KKGK++EI K K+PT+LKK+ILKER+E+
HHP  : 61  FHTKDSTNRKPLTKSQPCLTSFNSVDIASSKAKKGKEKEIAKLKRPTALKKVILKEREEK 120

SBP2 : 535 MQQRLQE----SAVSPT-----VASDDSQDVESGVTNQIPSP-DNPTGPEKTEEPMSSTP 584
                     +  PT        D  Q++ S     +  P D    P     P   TP
HHP  : 121 KGRLTVDHNLLGSEEPTEMHLDFIDDLPQEIVSQEDTGLSMPSDTSLSPASQNSPYCMTP 180

SBP2 : 585 VVEGESEEPAGTEFQRDPEACQPAPDSATFPKIHSRRFRDYCSQMLSKEVDACVTGLLKE 644
           V +G    PA +     P A      S+T  KIHS+RFR+YC+Q+L KE+D CVT LL+E
HHP  : 181 VSQGS---PASSGIG-SPMA------SSTITKIHSKRFREYCNQVLCKEIDECVTLLLQE 230

SBP2 : 645 LVRFQDRMYQKDPVKAKTKRRLVLGLREVLKHLKLRKLKCIIISPNCEKTQSKGGLDDTL 704
           LV FQ+R+YQKDPV+AK +RRLV+GLREV KH+KL K+KC+IISPNCEK QSKGGLD+ L
HHP  : 231 LVSFQERIYQKDPVRAKARRRLVMGLREVTKHMKLNKIKCVIISPNCEKIQSKGGLDEAL 290

SBP2 : 705 HTIIDCACEQNIPFVFALNRKALGRSLNKAVPVSIVGIFSYDGAQDQFHKMVELTMAARQ 764
           + +I  A EQ IPFVFAL RKALGR +NK VPVS+VGIF+Y GA+  F+K+VELT  AR+
HHP  : 291 YNVIAMAREQEIPFVFALGRKALGRCVNKLVPVSVVGIFNYFGAESLFNKLVELTEEARK 350

SBP2 : 765 AYKTMLKTMRQEQAGEPGPQTPPSPPMQDPIQSTDEG---TLASTGEEPHYIEIWRKHLE 821
           AYK M+  M QEQA E                +       +  S   EP   E+  K  E
HHP  : 351 AYKDMVAAMEQEQAEEALKNVKKVPHHMGHSRNPSAASAISFCSVISEP-ISEVNEKEYE 409

SBP2 : 822 AYSQHALELEDSLEASTSQ 840
             ++ +E  D LEAS ++
HHP  : 410 TNWRNMVETSDGLEASENE 428
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**Fig. 1.** Predicted amino acid sequence of SBP2. (**A**) The largest ORF of the isolated SBP2 cDNA encodes a protein of 846 amino acids with a predicted molecular mass of 94.86 kDa. The positions of six peptides obtained by LC/MS analysis are underlined. Asterisks are placed above the amino acids conserved in the putative RNA binding domain. The putative nuclear localization signal is boxed. (**B**) BLASTp-generated alignment of SBP2 and human hypothetical protein KIAA0256 (HHP).

reduce significantly both SBP2 binding and translational readthrough of a UGA codon (Lesoon *et al*., 1997). We believe the lower molecular weight species detectable in this cross-linking assay to be degradation products formed during the assay procedure as they were detected intermittently during purification (P.R.Copeland and D.M.Driscoll, unpublished data). No specific binding activity was detectable in bacterial extracts that were derived from non-induced cultures.

Having established that the RNA binding domain of SBP2 resides in the C-terminal two-thirds of the protein, we generated polyclonal antibodies to the bacterially expressed C-terminal domain. Using these antibodies, we specifically immunoprecipitated a 120 kDa protein from rat testis extracts (Figure 3B). An anti-peptide antibody raised against an N-terminal peptide (amino acids 179–197) also specifically immunoprecipitated SBP2 from a UV cross-linking reaction. In this experiment either S100 extracts or partially purified SBP2 were UV cross-linked to $^{32}$P-labeled PHGPx 3′ UTR. Anti-peptide SBP2

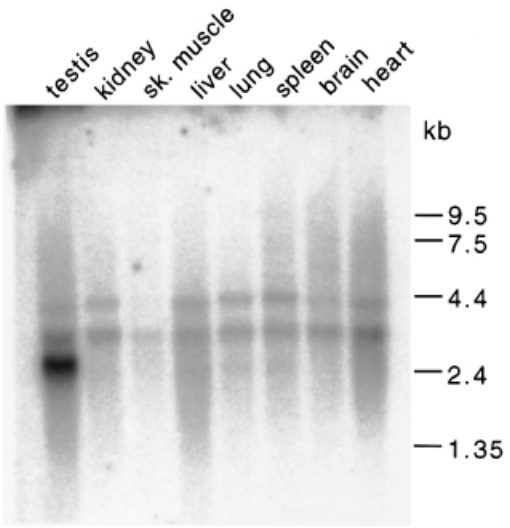


**Fig. 2.** Tissue distribution of SBP2 mRNA. A multiple tissue Northern blot (Clontech) of poly(A)$^+$ RNAs from rat tissues was probed with $^{32}$P-labeled SBP2 cDNA. Molecular weight standards are indicated on the right.

antibodies or pre-immune sera were added to these reactions and the immune complexes were precipitated on protein A–agarose. Figure 3C shows that in each case only the immune serum immunoprecipitated the 120 kDa cross-linked band.

### *In vivo targets of SBP2*

In an attempt to show an *in vivo* interaction between SBP2 and selenoprotein mRNAs, we immunoprecipitated SBP2 from extracts derived from a rat hepatoma cell line (McArdle 7777) in which both PHGPx and SBP2 are known to be expressed (Copeland and Driscoll, 1999). RNA was extracted from the immunoprecipitated protein and identified by reverse transcription followed by the polymerase chain reaction (RT–PCR). Using primers specific for PHGPx amplification, the expected ~800 bp band was obtained from the complexes immunoprecipitated with immune serum but not with pre-immune serum (Figure 4, top panel). Consistent with our previous data that demonstrated SBP2 binding to the GPx 3′ UTR, we were also able to amplify the expected fragment using GPx-specific primers as well (Figure 4, middle panel). No products were obtained in the absence of reverse transcriptase, thus eliminating the possibility of DNA contamination. As a control, a PCR specific for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also performed. In this case only a very faint band was obtained in both pre-immune and immune serum immunoprecipitations, suggesting that a small amount of this abundant RNA was non-specifically trapped during the procedure (Figure 4, bottom panel).

### *Analysis of Sec incorporation in vitro*

Although the fact that SBP2 interacts with selenoprotein mRNAs *in vivo* is compelling evidence for its role in selenocysteine incorporation, we set out to develop a reliable *in vitro* system to study selenoprotein synthesis directly. We have used a modified procedure for translation in reticulocyte lysates to demonstrate Sec insertion using $^{75}$Se-labeled Sec-tRNA$^{Sec}$. A diagram of the constructs

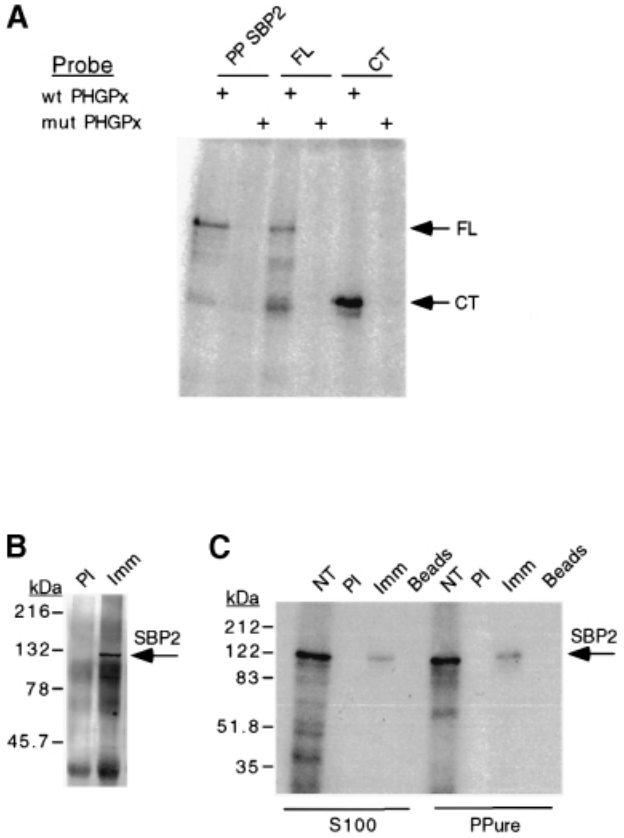


**Fig. 3.** UV cross-linking and immunoprecipitation of endogenous and recombinant SBP2. (**A**) Partially purified (PP SBP2) and recombinant Strep-tagged full-length (FL) or C-terminal (CT) SBP2 were incubated with either wild-type (wt) or mutant (mut) $^{32}$P-labeled PHGPx 3′ UTRs and subjected to UV cross-linking. After digestion with RNase A, the proteins were separated by SDS–PAGE and visualized by autoradiography. (**B**) Pre-immune (PI) and immune (Imm) sera raised against recombinant SBP2 were added to 1 mg of rat testicular S100 extract and complexes were precipitated with protein A–agarose. Proteins were resolved by electrophoresis, blotted to PVDF membrane and probed with anti-SBP2 peptide antiserum. (**C**) UV cross-linking assays were performed as described in (A) using 30 μg of S100 testicular extracts or 3 μg of partially purified SBP2 (PPure). Anti-SBP2 peptide antibody was added to the reactions as indicated, and complexes were precipitated with protein A–agarose and resolved by electrophoresis. Non-antibody-treated samples (NT) and samples treated with protein A–agarose alone (beads) were included as controls. Radiolabeled proteins were detected by PhosphorImager analysis.

used is presented in Figure 5A. These include PHGPx constructs that contained either a UGA (Sec), UGU (Cys) or UAA (Stop) codon at the Sec insertion site, and either wild-type or point-mutant (pt-mut; AUGA→ACGA) 3′ UTRs as indicated. We first optimized the conditions where PHGPx synthesis was only obtained when the in-frame stop codon specified Sec and the 3′ UTR was wild type. Under standard *in vitro* translation conditions (mRNA concentration of ~4 μg/ml, incubation at 30°C), no SECIS element-dependent translation was observed. This optimum was obtained only when small quantities of mRNA (0.4 μg/ml) were used to program the lysate, and when the reaction was incubated at 37°C (data not shown).

We used the set of PHGPx constructs shown in Figure 5A to program reticulocyte lysates in the presence of $^{75}$Se-labeled Sec-tRNA$^{Sec}$ purified from HL60 cells.

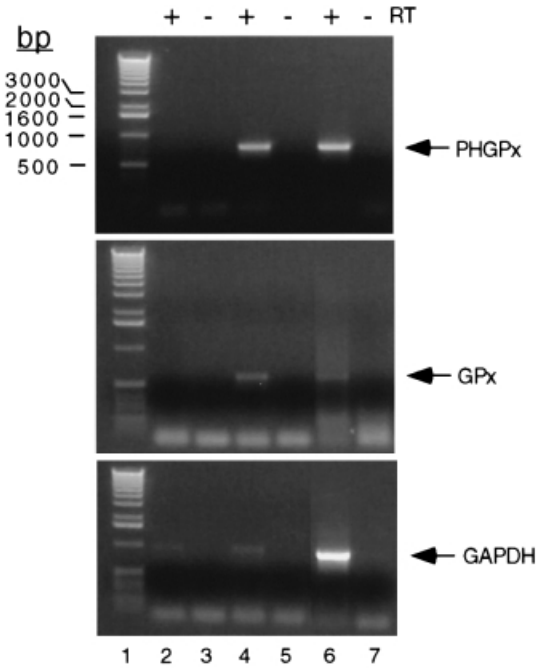


**Fig. 4.** Interaction of SBP2 with selenoprotein mRNAs *in vivo*. Pre-immune or anti-SBP2 antisera were added to cytoplasmic extracts from McArdle 7777 cells, and complexes were precipitated with protein A–agarose. The co-immunoprecipitated RNAs were extracted and used as templates for RT–PCR using PHGPx- (top), GPx- (middle) or GAPDH-specific primers (bottom). Reverse transcriptase (RT) was included as indicated. RT–PCR from immune precipitated beads is shown in lanes 4 and 5, that from pre-immune beads in lanes 2 and 3. RT–PCR from total McArdle RNA is shown in lanes 6 and 7. The PCR products were resolved by 1% agarose gel electrophoresis. Lane 1 contains a 1 kb DNA ladder.

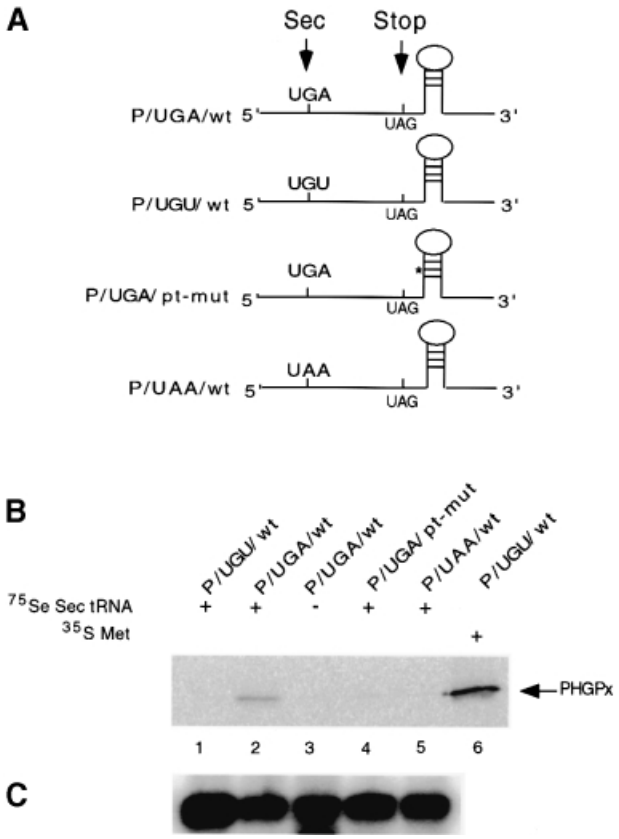


**Fig. 5.** Sec incorporation *in vitro*. (**A**) Diagram of the PHGPx constructs used in this study. (**B**) PHGPx mRNAs shown in (A) were added to reticulocyte lysates in the presence of $^{75}$Se-labeled Sec-tRNA$^{Sec}$ or [$^{35}$S]Met as indicated. *In vitro* translated PHGPx protein was purified by BSP–agarose treatment, resolved by electrophoresis and detected by PhosphorImager analysis. $^{35}$S-labeled PHGPx is included as a control for PHGPx migration during electrophoresis (lane 6). (**C**) Wild-type and mutant PHGPx mRNAs were extracted at the end of *in vitro* translation reactions and analyzed by Northern blotting ($n$ = 3). A representative experiment is shown.

PHGPx protein was purified from the reaction by affinity chromatography on bromosulfophthalein–glutathione–agarose (BSP–agarose) and analyzed by SDS–PAGE and PhosphorImager analysis. As no other radioactive label was present in the reaction, any signal obtained is the result of $^{75}$Se-labeled Sec incorporation into PHGPx protein. As shown in Figure 5B, PHGPx translation was only detectable when the UGA codon specified Sec in the context of the wild-type PHGPx 3′ UTR (lane 2). No incorporation was detectable when the Sec codon is changed to UGU or UAA or when it is placed in the context of a mutant UTR (Figure 5B, lanes 1, 5 and 4, respectively). Figure 5C shows Northern blot analysis of PHGPx mRNAs extracted at the end of an *in vitro* reaction. Similar levels of both wild-type and mutant mRNAs were recovered, indicating that differential mRNA stability does not play a role in the translational regulation of PHGPx mRNA.

### *Effect of SBP2 on PHGPx translation*

We also tested $^{75}$Se-labeled Sec incorporation in the presence of *in vitro* translated SBP2. For this experiment, RNAs encoding full-length, N-terminal amino acids 1–284 or C-terminal amino acids 280–846 of SBP2 were pre-translated in reticulocyte lysate. After a 15 min incubation at 30°C, the PHGPx mRNAs shown in Figure 5A were added to the reaction and incubation was continued for 50 min at 37°C. Figure 6A shows the SBP2 translated products obtained by the end of the reaction. Figure 6B shows that in the presence of either full-length or the C-terminal fragment of *in vitro* translated SBP2, PHGPx synthesis is significantly enhanced, suggesting that this factor is limiting in reticulocyte lysates. This point is further demonstrated by the relative overexpression of the C-terminal fragment compared with the full-length SBP2 (Figure 6A), which is consistent with the greater enhancement of PHGPx translation in the presence of this form of SBP2 (Figure 6B). No enhancement is observed, however, in the presence of an N-terminal fragment of SBP2, which was generated from a truncated mRNA. It is notable that SBP2 addition stimulates PHGPx synthesis even when the 3′ UTR harbors an SECIS element mutation (P/UGA/pt-mut construct, Figure 6B). Quantitative analysis of this experiment indicates that the expression of the C-terminal fragment of SBP2 stimulates PHGPx synthesis by 25-fold, and that this stimulation is 3.6-fold less efficient for the SECIS element mutant. Since the SECIS point mutant did not completely abrogate the effect of SBP2, we chose to analyze a more deleterious PHGPx construct, which possesses a deletion of the entire AUGA element (Δ-mut). This mutant was translated in the presence of C-terminal SBP2, and as shown in Figure 6C no readthrough is detectable when the AUGA element is deleted, indicating that the stimulatory effect is entirely

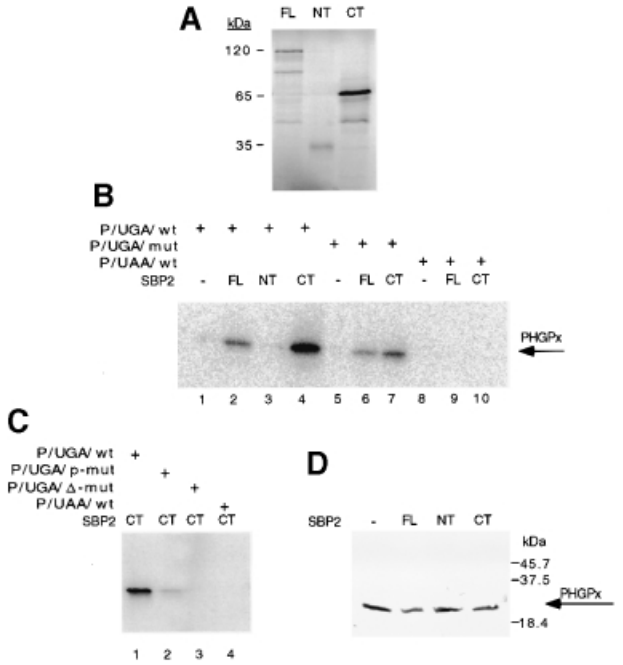


**Fig. 6.** Addition of SBP2 to reticulocyte lysates. (**A**) Full-length (FL), N-terminal (NT) and C-terminal (CT) SBP2 mRNAs were translated in reticulocyte lysates in the presence of [$^{35}$S]Met. Reactions were terminated, and proteins were resolved by SDS–PAGE. (**B**) PHGPx mRNAs as shown in Figure 5A were translated in reticulocyte lysates in the presence of $^{75}$Se-labeled Sec-tRNA$^{Sec}$ and in the presence or absence of the SBP2 proteins described in (A). *In vitro* translated PHGPx was purified on BSP–agarose and resolved by SDS–PAGE. (**C**) PHGPx mRNAs as indicated were translated in reticulocyte lysates as described in (B) except that all reactions were performed in the presence of pre-translated C-terminal SBP2. (**D**) As in (B) except that *in vitro* translated PHGPx protein (from P/UGU/wt construct) was blotted to PVDF and probed with anti-PHGPx antibody.

3′ UTR dependent. The stability of the Δ-mut is not altered relative to the wild-type mRNA as determined by Northern analysis (data not shown). Figure 6D shows that SBP2 did not enhance the translation of the Sec→Cys mutant (P/UGU/wt) even in the presence of a wild-type 3′ UTR.

### *SBP2 is required for Sec insertion in vitro*

Using the assay described above, we also tested whether SBP2 is required for the basal level of Sec insertion in reticulocyte lysate. To deplete SBP2 from reticulocyte lysates, anti-SBP2 polyclonal antibody and a pre-immune control were used to immunoprecipitate SBP2 from the lysates. The protein–antibody complexes were removed with protein A–agarose and the resulting depleted lysates analyzed for their ability to synthesize the PHGPx constructs shown in Figure 5A in a codon- and UTR-specific manner. Figure 7 shows that the anti-SBP2 antibody treatment completely eliminates PHGPx translation. When *in vitro* translated SBP2 is added back to these lysates, PHGPx translation is dramatically enhanced. The depletion of SBP2 had no effect on the translation of non-selenocysteine-containing messages (data not shown). These results clearly demonstrate that SBP2 is required for selenoprotein synthesis *in vitro*.

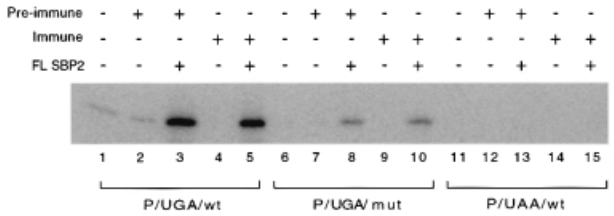


**Fig. 7.** SBP2 is required for Sec incorporation in reticulocyte lysates. Reticulocyte lysates were treated with either pre-immune or anti-SBP2 immune sera, and complexes were precipitated with protein A–agarose. PHGPx mRNAs as shown in Figure 5A were translated in the depleted reticulocyte lysate in the presence of $^{75}$Se-labeled Sec-tRNA$^{Sec}$. Full-length SBP2 was added back to depleted lysates as indicated. Proteins were resolved by SDS–PAGE and detected by PhosphorImager analysis.

## Discussion

Based on transfection and *in vitro* experiments, Sec incorporation is an inefficient process that is likely to be limited by three converging pathways: Sec-tRNA$^{Sec}$ biosynthesis, SECIS binding protein–elongation factor interactions, and competition with translation termination. While the latter of these has been shown to be the major limitation in bacterial Sec incorporation (Suppmann *et al*., 1999), the interplay between these systems in eukaryotes remains to be determined. In this study we have demonstrated that a SECIS binding protein is a necessary component of the Sec insertion machinery, and in the context of reticulocyte lysates it is limiting. We have identified the cDNA encoding this novel protein, and demonstrated its binding specificity and function in a newly developed *in vitro* translation assay for selenoprotein biosynthesis.

### *SBP2 primary structure*

As illustrated in Figure 1B, the C-terminal 429 amino acids of SBP2 have 46% identity with a protein of unknown function (human hypothetical protein KIAA0256, DDBJ/EMBL/GenBank accession No. D87445). The most striking feature in the SBP2 sequence is in a region found to be similar to a putative RNA binding motif found in many ribosomal proteins as well as the SUP1 suppressor of translation termination (Koonin *et al*., 1994). Interestingly, the identity between SBP2 and KIAA0256 is 75% in a 60-amino-acid region surrounding this motif. While no data exist concerning the role of this motif in binding RNA or interacting with the termination machinery, it stands out as a promising starting point for a structure–function analysis of SBP2.

The predicted amino acid sequence of SBP2 is also notable in that it contains several stretches of consecutive lysine residues, which are predicted to function as nuclear localization signals. As all of our analyses of SBP2 have utilized cytoplasmic extracts, this brings up the intriguing possibility that the subcellular localization of SBP2 is regulated and that it may serve some other function as a nuclear protein.

### *Expression of SBP2*

Northern analysis of poly(A)$^+$ RNA from various tissues reveals that SBP2 may be encoded by as many as three distinct transcripts. Interestingly, the smallest transcript (2.5 kb) is only detectable in testis, and its abundance

correlates with the large excess of PHGPx mRNA present in testis compared with other tissues (Pushpa-Rekha *et al*., 1995). The expression of the small transcript also correlates with the abundance of SBP2 protein, which was easily detectable in testis, but only barely detectable in liver and undetectable in spleen and kidney (P.R.Copeland and D.M.Driscoll, unpublished data). The size of the over-expressed mRNA can only account for the coding region of SBP2 without extensive untranslated regions. It is possible, therefore, that the added sequences in the larger forms of SBP2 mRNA are involved in regulating its expression or encoding proteins with other functions. Further analysis of the untranslated regions as well as searching for alternatively spliced variants should help to shed light on the complexities of SBP2 expression.

### *SBP2 is required for Sec incorporation*

A significant advance in the analysis of selenoprotein biosynthesis is found here in our effective use of reticulocyte lysates in combination with $^{75}$Se-labeled Sec-tRNA$^{Sec}$ to analyze Sec incorporation *in vitro*. *In vitro* translation of the deiodinase 1 protein was reported in 1991, but it was not shown to be SECIS element dependent and may have been the result of non-specific suppression of the UGA codon (Berry *et al*., 1991b). SECIS element-dependent *in vitro* translation was reported later that year by the same group, but instead of direct Sec incorporation, [$^{35}$S]Met incorporation was analyzed (Berry *et al*., 1991a). Further analysis of the *in vitro* reaction revealed that nonsense suppression yielded the predominant product during GPx translation in reticulocyte lysates. Inclusion of the Sec-tRNA$^{Sec}$ in this experiment prevented suppression and eliminated UGA readthrough, suggesting that the tRNA was preventing the utilization of the standard elongation factor (EF1A) and was in position for programmed insertion, but that the system was depleted of other required factors (Jung *et al*., 1994).

The key difference between the published use of reticulocyte lysates and the method described here is the amount of mRNA used to program the reaction, and the incubation temperature. In our analysis of PHGPx translation *in vitro*, the use of standard amounts of mRNA (4 μg/ml) resulted in substantial amounts of non-SECIS-dependent product formation that were presumably the result of nonsense suppression (P.R.Copeland, J.E.Fletcher and D.M.Driscoll, unpublished data). This suggests that the higher concentration of mRNA in the reaction was diluting multiple components in the lysate that must interact with the mRNA to allow Sec insertion. Our previous analysis of the binding characteristics of SBP2 indicated that the optimal binding temperature was between 30 and 37°C (Copeland and Driscoll, 1999). Consistent with this finding is the fact that Sec incorporation *in vitro* is more than twice as efficient at 37°C than at 30°C. Using this system in combination with anti-SBP2 antibodies, we have demonstrated that SBP2 is a required component of the Sec insertion machinery, and that the C-terminal two-thirds of the protein possess both RNA binding activity as well as full Sec incorporation activity.

### *On the mechanism of SBP2 function*

Our results demonstrate that SBP2 is critical in the translation of selenoprotein mRNAs, but two lines of evidence suggest that SBP2 is not acting alone on the SECIS element. First, gel filtration analysis of SBP2 binding activity indicated an aggregate molecular mass of ~500 kDa (Copeland and Driscoll, 1999). Secondly, SBP2 binding activity is not affected by mutations in the tetra-adenosine element in the terminal region of the PHGPx 3′ UTR, yet these same mutations completely eliminate Sec incorporation (Lesoon *et al*., 1997). It is likely, therefore, that this region of the SECIS element is interacting with some other factor—perhaps directly with the ribosome or Sec-tRNA$^{Sec}$. Based upon the putative RNA binding domain found in SBP2, which is associated with suppressing translation termination in yeast, we propose that SBP2 might be primarily involved in preventing the termination reaction. SBP2 may be part of a multiprotein complex, the other members of which are directly involved with delivering Sec-tRNA$^{Sec}$ to the ribosome A site during a pause at the UGA codon mediated by SBP2. The development of an *in vitro* system and the identification of SBP2 described here should significantly enhance the pace at which other components of the Sec insertion machinery can be identified and tested.

## Materials and methods

### *Plasmid construction and RNA synthesis*

For the PHGPx constructs, mutant 3′ UTRs (Lesoon *et al*., 1997) were subcloned into the *Sma*I and *Bsg*I sites of pCR3.1 vector containing the cytoplasmic (short) form of the PHGPx coding region. The *Pst*I–*Hin*dIII fragment containing the entire PHGPx sequence was blunted and subcloned into the *Sma*I site of pT7A63 (a gift of M.Wormington), which contains a 3′ poly(A) tail 63 nt in length. To change the PHGPx Sec codon to UAA, site-directed mutagenesis was performed using the Altered Sites Mutagenesis kit (Promega) with the mutagenic oligonucleotide GGCCTCGCAA**TA**AGGCAAAACCG (altered nucleotides in bold). PHGPx constructs were linearized with *Xba*I prior to transcription. The wild-type and mutant PHGPx 3′ UTR RNAs used for UV cross-linking analysis were prepared as described (Lesoon *et al*., 1997). Full-length and C-terminal SBP2 (see below) mRNAs were generated by linearizing with *Xba*I. Strep-tagged full-length SBP2 was generated according to the manufacturer's protocol (Genosys) using the following 5′ and 3′ primers, respectively: CGATTATGGTCTCAGCGCGCGTCGGAGCGG-CCGCGGGAG and CGATTATGGTCTCCTATCAACGGGAGGTCT-GGGTGTCTG. Strep-tagged C-terminal SBP2 was made by ligating an *Eco*RI–*Xho*I fragment of EST H31811 into *Eco*RI–*Xho*I-digested pASK-IB7 (Genosys). With the exception of the UV cross-linking substrate RNAs, all *in vitro* transcription (Ribomax T7, Promega) was performed in the presence of m$^7$GpppG (Roche) for the synthesis of capped mRNA. All plasmid constructs were verified by DNA sequencing.

### *Protein purification and peptide sequencing*

SBP2 purification was performed as described (Copeland and Driscoll, 1999). For protein sequencing, three preparations of RNA affinity-purified SBP2 were combined and electrophoresed in an 8% SDS–PAGE gel followed by Coomassie staining and destaining. The stained gel was sent to the W.M.Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia for capillary LC/MS peptide sequencing. The purified protein was excised from the gel and digested *in situ* with trypsin (Shevchenko *et al*., 1996). The digest was analyzed by capillary LC-electrospray MS, and peptide amino acid sequences were characterized by collisionally activated dissociation (CAD) using LC-electrospray-tandem MS. The peptide amino acid sequences were determined by manual interpretation of the CAD spectra. In order to obtain additional sequence information, the digest was fractionated by capillary HPLC so that selected peptides could be analyzed by tandem MS following N-terminal derivatization with 2-(3-pyridyl) ethanoic acid *N*-hydroxysuccinimide ester.

### *SBP2 cloning*

Peptide sequences were used to query the EST and non-redundant DDBJ/EMBL/GenBank databases using the tBLASTn program. The peptide

VPVQXDXGGFXAAXEK (where X = I or L) was identical to the predicted sequence of EST H31811 from rat PC-12 cells. The H31811 clone (in pBluescript SK$^-$, Stratagene) was obtained from ATCC and upon sequencing was found to contain a 1410 nt ORF and a 656 nt 3′ UTR containing a canonical nuclear polyadenylation site (AATAAA). To obtain the 5′ portion of the SBP2 clone, 5′ RACE was performed using a primer based on H31811 sequence (GCATCTTCAATCCTC-GGTGGCT) against DNA purified from an amplified λgt11 rat testis cDNA library (Clontech), and the products cloned into pCR2.1 (Invitrogen). Three clones were obtained that overlapped with H31811 sequence, the longest of which (2.2 kb) was used for further analysis. The 5′ RACE clone contained an ORF of 1118 nt as well as >1 kb of upstream sequence. Since this upstream sequence contained an *Eco*RI site (the site used in library construction) 28 nt upstream from the ORF ATG and contained an unrelated ORF, we have discarded that sequence as it is likely to be a chimeric clone. To obtain full-length SBP2 cDNA, PCR was performed on rat spleen Marathon cDNA (Clontech) to isolate a 1349 bp fragment that overlapped the 5′ RACE and EST H31811 clones. This fragment was digested with *Bsa*MI and *Bsr*GI, and combined with *Bsr*GI–*Kpn*I-digested 5′ RACE clone and *Bsa*MI–*Kpn*I-digested H31811 in a triple ligation reaction. The SBP2 clone used in this study was obtained by PCR with a start-site primer and a 3′ primer ending immediately upstream of the polyadenylation site (ATGGCGTCGG-AGCGGCCGC and CTAACAAATAAGCCCTCTTGC, respectively), which generated a 3173 bp fragment.

### Expression of recombinant proteins

Full-length and C-terminal SBP2 constructs in pASK-IB7 were transformed into *Escherichia coli* BL21 (Promega). Transformed bacteria were grown in LB medium to a density of 0.5–0.7 $OD_{550}$, then induced with anhydrotetracycline (0.2 mg/ml) for 2.5 h at 30°C. The cells were pelleted, resuspended in Buffer W (100 mM Tris–HCl pH 8.0, 1 mM EDTA) with protease inhibitors (Complete, Roche) and lysed by freeze–thaw, followed by sonication for 15 s/ml. The sonicate was then centrifuged at 10 000 *g* for 15 min at 4°C. The supernatant was applied directly to a 0.5 × 10 cm column containing Streptactin-POROS equilibrated in Buffer W with 25 mM KCl and tagged protein was eluted in Buffer W containing 2.5 mM desthiobiotin (Sigma).

### Western blot analysis, immunoprecipitation and immunodepletion

Polyclonal anti-SBP2 antibodies were raised in rabbits against Streptactin purified C-terminal SBP2 as well as a synthetic peptide sequence found in the N-terminal region corresponding to amino acids 179–197 (CHLKSDGYHKRPDRKSRIL; Bio Synthesis). Immunoprecipitations were performed with the indicated amounts of anti-protein antibody or anti-peptide antibody and protein A–agarose (Roche). Western analysis was performed with a 1:500 dilution of anti-peptide antibody and developed using the Protoblot alkaline phosphatase detection system (Promega). For immunoprecipitation from tissue extracts, antibodies were coupled to protein A–agarose with dimethylpimelimidate as described (Harlow and Lane, 1988) using NET-2 buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.05% NP-40). For immunodepletion of reticulocyte lysates, a 1:50 dilution of anti-SBP2 antibody or pre-immune serum was added to the lysates, incubated for 1 h at 4°C, then added to 20 µl of protein A beads pre-equilibrated in phosphate-buffered saline (PBS) and mixed at 4°C overnight. Translation reactions were carried out as described below.

### UV cross-linking analysis

UV cross-linking assays were carried out exactly as described using the indicated amounts of S100 rat testicular extract or partially purified SBP2 (Copeland and Driscoll, 1999).

### Immunoprecipitation of SBP2 target RNAs

McArdle 7777 cells were lysed directly on a 55 $cm^2$ plate in 500 µl of lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 1% Triton X-100) and pulled through a 25 gauge syringe needle 10 times followed by centrifugation at 14 000 *g*. Total protein (1 mg) from the resulting cytoplasmic extract was treated with a 1:250 dilution of anti-SBP2 antibody or pre-immune serum. SBP2 and associated RNAs were immunoprecipitated essentially as described (Shih and Claffey, 1999). Protein A beads were washed in lysis buffer and RNA was extracted directly from the beads in 250 µl of Trizol (Life Technologies, Inc.) according to the manufacturer's protocol. Precipitated RNAs were resuspended in 10 µl of water and 1 µl was used in a 20 µl reverse transcriptase reaction (AMV; Life Sciences, Inc.). Immunoprecipitated RNAs were identified by PCR using primers specific for PHGPx, GPx or GAPDH. PHGPx primers (CCGCTTATTGAAGCC-AGCACTGC and CCCACAAGGCAGCCAAGGTGAA) amplify a 772 bp fragment, GPx primers (ATGTCTGCTGCTCGGCTCTCCGC and GGGGTTGCTAGGCTGCTTGGACA) amplify a 603 bp fragment, and GAPDH primers (Clontech) amplify an ~1 kb fragment. PCRs were amplified for 35 cycles, annealing at 55°C for 30 s and extending for 1 min at 72°C. Products were resolved on 1% agarose gels and their sizes determined by comparison to a 1 kb ladder standard (Life Technologies, Inc.).

### Preparation of Sec-tRNA$^{Sec}$

$^{75}$Se-labeled Sec-tRNA$^{Sec}$ was prepared using HL60 cells (Lee *et al*., 1989; Jung *et al*., 1994) and the two isoacceptors (mcmUm and mcmU) were resolved from each other by RPC-5 chromatography as described (Hatfield *et al*., 1991). The specific activity of the $^{75}SeO_2^-$ as $H_2SeO_3$ was 190 mCi/mmol. For the experiments described here, the two isoacceptor fractions were pooled and 5500 c.p.m. were used per reaction.

### Northern blot analysis

A rat multiple tissue Northern blot (Clontech) was probed with an [α-$^{32}$P]dCTP random primed SBP2 probe (RadPrime; Life Technologies, Inc.) corresponding to the EST H31811 sequence using Expresshyb hybridization solution and conditions. The washed membrane was exposed to a PhosphorImager screen (Molecular Dynamics).

### In vitro translation

Capped, polyadenylated PHGPx mRNAs were generated by Ribomax T7 (Promega) *in vitro* transcription from *Xba*I-linearized plasmid DNAs. Capped non-polyadenylated SBP2 RNAs were made similarly except that for full-length and C-terminal mRNAs the respective plasmids were linearized with *Xba*I, and for the SBP2 N-terminal fragment mRNA the plasmid containing full-length SBP2 was linearized with *Bsr*GI. All *in vitro* translation reactions utilized rabbit reticulocyte lysates (70% v/v; Promega) with the following additives: 20 µM complete amino acid mix, 0.8 U/µl RNA Guard (Pharmacia), 0.4 µg/ml PHGPx RNA and 5500 c.p.m. $^{75}$Se-labeled Sec-tRNA$^{Sec}$. For the addition of SBP2 protein, SBP2 mRNAs encoding either the full-length, N-terminal or C-terminal portions were pre-translated in the reticulocyte lysate reactions described above at a concentration of 4 µg/ml. Reactions were incubated at 30°C for 15 min to allow SBP2 translation prior to adding PHGPx mRNAs, which were further incubated at 37°C for 50 min. Total reaction volumes ranged from 12 to 50 µl. For a 50 µl reaction, 25 µl were added to 255 µl of PBS containing 2 mM dithiothreitol (DTT) and 25 µl of BSP–agarose (Sigma). This mixture was incubated for 1 h at 4°C, then the beads were washed with PBS–DTT and the protein eluted in SDS sample buffer, followed by separation using 15% SDS–PAGE. Radiolabeled PHGPx was detected by PhosphorImager analysis. The remainder of the *in vitro* translation reaction was used for Northern analysis as described above. For determining RNA stability, RNA was extracted from 25 µl of the translation reactions used for Figure 5 using Trizol (Life Technologies, Inc.) according to the manufacturer's protocol. Resuspended RNAs were denatured with formaldehyde and formamide, and separated by electrophoresis in 1.2% agarose, 2 M formaldehyde gels. Gels were transferred to nylon membranes, which were subsequently probed with an [α-$^{32}$P]dCTP-labeled PHGPx cDNA probe prepared by random primer extension.

### DDBJ/EMBL/GenBank data

The DDBJ/EMBL/GenBank accession number for *Rattus norvegicus* mRNA for SECIS binding protein 2 (*sbp2* gene) is AJ251245.

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