Review

Jonathan N. Gonzalez-Flores, Sumangala P. Shetty, Aditi Dubey and Paul R. Copeland*

The molecular biology of selenocysteine

Abstract: Selenium is an essential trace element that is incorporated into 25 human proteins as the amino acid selenocysteine (Sec). The incorporation of this amino acid turns out to be a fascinating problem in molecular biology because Sec is encoded by a stop codon, UGA. Layered on top of the canonical translation elongation machinery is a set of factors that exist solely to incorporate this important amino acid. The mechanism by which this process occurs, put into the context of selenoprotein biology, is the focus of this review.

Keywords: elongation factor; RNA binding; SECIS binding protein; selenocysteine; translation.

***Corresponding author: Paul R. Copeland,** Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA,
e-mail: copelapr@umdnj.edu
Jonathan N. Gonzalez-Flores, Sumangala P. Shetty and Aditi Dubey: Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA

Introduction: selenoprotein synthesis

Selenium is a micronutrient and an antioxidant in the form of selenocysteine

Selenium was first discovered to be an essential trace element in the 1950s by the German scientist Klaus Schwarz. Experiments with vitamin E-deficient rats subjected to an exclusive diet of torula yeast showed that they developed liver necrosis, but they were normal when fed with brewer's yeast (1). The unknown dietary component in the brewer's yeast was called 'factor 3', and it was associated with two additional factors that were previously discovered to prevent dietary liver necrosis: factor 1, which was sulfur-containing amino acids, and factor 2, which was vitamin E (1, 2). Further analysis revealed that fractions of factor 3 that alleviated liver necrosis in rats were enriched for selenium (3). Rats fed the torula yeast diet supplemented with trace amounts of sodium selenate were protected against liver necrosis, therefore confirming selenium as an essential micronutrient in mammals (3).

Selenium was first recognized as an antioxidant from experiments performed by Rotruck and coworkers in rat erythrocytes (4). In this study, selenium protected the cellular membrane and hemoglobin of erythrocytes against oxidative damage through the utilization of glutathione (GSH). GSH is an essential cofactor of the GSH peroxidase (GPX) for the catalytic breakdown of H_2O_2 and lipid peroxides (5). Interestingly, the enzymatic activity of GPX was selenium dependent (6, 7). Other reports also mentioned selenium as an important factor for the activity of *Escherichia coli* formate dehydrogenase (8) and *Clostridium sticklandii* glycine reductase (9). The catalytic role of selenium in these enzymes was proposed to be either as a cofactor, as a reaction intermediate, or as a covalent moiety (7). By 1976 the research group of Thressa C. Stadtman had discovered that selenium was covalently attached in the *C. sticklandii* glycine reductase as a selenocysteine (Sec) residue (10). This finding was also confirmed in GPX where the catalytic site was identified as Sec (11). The presence of Sec was also confirmed in other proteins, classified as selenoproteins, in most organisms from all domains of life (12, 13).

Biology of selenoproteins

Computational and experimental approaches have identified 25 selenoproteins in the human proteome (14). These selenoproteins can be classified according to their biological function into six different groups: 1) peroxidase and reductase activities, 2) hormone metabolism, 3) protein folding, 4) redox signaling, 5) Sec synthesis, and 6) selenium transport (15, 16). Roughly half of selenoproteins (8 peroxidases/reductases and 4 redox signaling) confer cellular protection against oxidative stress (17–19). As a detailed consideration of each of these selenoproteins is outside the scope of this review, we will later focus on only one case, selenoprotein P (SelP), which poses interesting

questions related to the mechanisms of selenoprotein synthesis.

The origins and evolutionary selection of Sec in selenoproteins continues to be a highly debated topic in the selenium field. August Böck and colleagues proposed that UGA was a sense codon for Sec in the anaerobic world around 2–3 billion years ago (20). The authors argued that introduction of oxygen in the atmosphere selected against the oxygen-sensitive Sec and restricted Sec insertion at UGA codons. Because of its poor adoption as a sense codon, the UGA-Sec codon gradually evolved as a termination signal or in a few cases a codon for cysteine (Cys) (20–22).

In contrast, Gladyshev and Kryukov (2001) suggest that the lack of selenoproteins in most anaerobic organisms, in addition to the high retention and conservation of the UGA-Sec codon in vertebrates (23), suggests that Sec was a 'recent' addition rather than a genetic relic (24). Moreover, Sec insertion would have been a rare event or unavailable in early life forms because selenium abundance in the environment is ~200 000 times lower than that of sulfur, which is a component in methionine and Cys (25). By comparison, methionine and Cys are the least abundant amino acids with a genomic frequency of 2.4% and 0.78% in all forms of life, respectively (26).

Why Sec and not Cys?

The majority of selenoproteins require Sec for their catalytic activity (27). Mutagenic studies revealed a dramatic decrease in the enzymatic activity of rat thioredoxin reductase (TrxR) when Sec was replaced by Cys (28). Substitution of Sec with Cys is also detrimental in formate dehydrogenase H (FDH_H), type 1 iodothyronine deiodinase (DIO1), and GPX1 (29–31). However, non-Sec selenoprotein homologs where Sec is replaced by Cys are naturally found across all domains of life. In fact, higher plants and fungi are completely devoid of selenoproteins, and many of the activities are carried out by Cys-containing homologs (32, 33). How can Cys be active in these naturally occurring homologs and not in *bona fide* selenoproteins?

Gromer et al. provided key findings that suggest Cys 'activation' is determined by the protein microenvironment by comparing a Sec-containing TrxR with its respective Cys homolog (34). To understand the catalytic advantage of Sec, they compared the catalytic motif Gly-Cys-Sec-Gly (GCUG) of rat TrxR with the Ser-Cys-Cys-Ser (SCCS) motif of *Drosophila melanogaster* TrxR (DmTrxR). A variety of mutants in the SCCS motif were analyzed including GCCG, SCUS, and the GCUG mammalian motif. The mutant variants of $DmTrxR^{SCUS}$ and $DmTrxR^{GCUG}$ retained wild-type activity, thus indicating that the catalytic mechanism is independent of the microenvironment in the active site. The $DmTrxR^{GCCG}$ mutant had significantly lower enzymatic activity than wild type. These results implied that the flanking serines in the insect motif are critical for Cys activation. However, rat TrxR with the insect motif ($TrxR^{SCCS}$) did not achieve Sec-independent activity, suggesting additional Cys-activating determinants that are outside of the catalytic pocket (35).

All of this brings an intriguing question: Why did some selenoproteins maintain Sec while others evolved with Cys? What is the advantage of having an energetically expensive machinery for Sec synthesis and incorporation if Cys active sites are possible? Having this in mind, Hondal and Ruggles developed a new 'chemico-biological' hypothesis that posits a biological convenience rather than a chemico-enzymatic advantage as the reason for selecting Sec over Cys (22). This biological convenience, the authors argued, could be the ability to resist enzymatic inactivation by irreversible oxidation. Sec in selenoproteins ($R\text{-}Se^-$) is oxidized to R-SeOH and can readily be recycled back to $R\text{-}Se^-$, whereas Cys cannot be recycled once it is over-oxidized to $R\text{-}SO_2^-$ or $R\text{-}SO_3^-$. This rationale led the authors to infer that Sec in selenoproteins prevents over-oxidation and enzyme inactivation, whereas the Cys homologs, by virtue of their catalytic role and/or biological function, might not require resistance to irreversible oxidation.

Synthesis of Sec-tRNA^{Sec}

The existence of a selenium-laden tRNA was first observed in experiments performed in rat liver where radiolabeled [^{75}Se]selenite was associated with a tRNA-bound Sec residue (36). In a follow-up article, Hawkes and Tappel showed that GPX incorporated [^{75}Se]Sec when [^{75}Se]Sec-tRNA was added in cell-free extracts from rat liver (37). Cloning and DNA sequencing of the mouse GPX gene revealed that the translational incorporation of Sec occurred at the UGA stop codon (38). This clearly indicated that Sec is not a posttranslation modification but rather is inserted into nascent peptides at in-frame UGA codons (10, 39).

The tRNA for Sec was originally co-discovered by two independent groups as a seryl UGA suppressor tRNA (40) and as a phosphoseryl-tRNA (pSer-tRNA) (41). Later studies revealed that the opal suppressor seryl-tRNA (Ser-tRNA) was the precursor of pSer-tRNA (42). The $tRNA^{Sec}$ is not aminoacylated directly with Sec, but rather

the phosphate group from the pSer-tRNA is substituted for a selenium atom (39). The $tRNA^{Sec}$ is the largest tRNA in eukaryotes with 90 bases and is the sole tRNA for Sec (39, 43).

The transcription of the $tRNA^{Sec}$ gene (*Trsp*) by RNA polymerase III is different from other canonical tRNAs because it is regulated by three upstream promoters (TATA box, proximal sequence element, and distal sequence element) instead of Box A and Box B internal promoters (39, 44). The $tRNA^{Sec}$ transcript starts at the mature 5′ site and only gets processed at its 3′ end (45). The $tRNA^{Sec}$ contains four modified bases: two at the T arm, 1-methyladenosine (m^1A) and pseudouridine (Ψ), and two at the anticodon arm, N^6-isopentenyl-adenosine (i^6A) and 5-methoxycarbonylmethyl-uridine (mcm^5U). The m^1A is required for the synthesis of Ψ, a modification that governs the $tRNA^{Sec}$ tertiary structure (46). The i^6A modification is critical for Sec recoding efficiency (47). The mcm^5U is further methylated to 5-methylcarboxymethyl-uridine-2-*O*-methylribose (mcm^5Um) when intracellular selenium levels are sufficient (48, 49). The proposed role of the mcm^5Um Sec-$tRNA^{Sec}$ isoform is to specifically synthesize stress-related selenoproteins that are sensitive to selenium status, whereas the mcm^5U isoform is thought to serve the synthesis of housekeeping selenoproteins (48, 50, 51). The mechanism for differential tRNA utilization has not been deciphered.

The aminoacylation of $tRNA^{Sec}$ is a multistep process, requiring four enzymatic reactions. First, $tRNA^{Sec}$ is charged with serine by the canonical Ser-tRNA synthetase (SerRS) (52). Second, a pSer-tRNA kinase specifically phosphorylates the Ser-$tRNA^{Sec}$ and forms pSer, an ideal leaving group for selenium substitution (53). Finally, the conversion of pSer-$tRNA^{Sec}$ to Sec-$tRNA^{Sec}$ is composed of two coupled enzymatic reactions: 1) The active donor of selenium, selenophosphate, is synthesized from selenide by the selenophosphate synthetase 2 (SPS2) (54). 2) The Sec synthase exchanges the phosphate moiety of pSer-$tRNA^{Sec}$ for selenium from the selenophosphate donor to generate Sec-$tRNA^{Sec}$ (55).

Sec incorporation: a special recoding event

In 1986, August Böck and colleagues demonstrated that Sec was introduced into the bacterial selenoenzyme formate dehydrogenase (*fdh*) via a special recoding event at in-frame UGA codons (56). The same group subsequently isolated mutants of *E. coli* that were defective for formate dehydrogenase activity, and they identified four genes: *selA*, *selB*, *selC*, and *selD* (20). The genes *selA*, *selC*, and *selD* were involved in the synthesis of Sec-$tRNA^{Sec}$, whereas *selB* was required during the Sec recoding event (57). Genes in the Sec-$tRNA^{Sec}$ synthesis pathway were characterized as follows: *selC* for $tRNA^{Sec}$, *selD* for selenophosphate synthetase, and *selA* for Sec synthase (58–60). The *selB* gene encoded a protein (SelB) that had sequence homology to translation elongation factors (61). SelB was characterized as a guanosine triphosphate (GTP)/guanosine diphosphate (GDP) binding protein that stably interacted with the Sec-$tRNA^{Sec}$. Furthermore, the translation elongation factor Tu (EF-Tu), the main carrier for aminoacyl-tRNAs (aa-tRNAs), did not recognize the Sec-$tRNA^{Sec}$ (62). These studies implicated SelB as the Sec-specific elongation factor for Sec-$tRNA^{Sec}$. Having identified the key players in the recognition of UGA as Sec, the question then became how the cell can differentiate between stop and Sec codons. An RNA folding program predicted a 40-base stem-loop structure immediately at the 3′ side of the Sec-UGA codon, which turned out to be essential for Sec incorporation (63). This finding solved the specificity problem and presented a complete set of factors that could alter the coding potential of specific UGA codons in bacteria.

At about the same time, the specificity of mammalian Sec incorporation was also being investigated, and it was found that the cis-acting specificity element was not in the coding region but instead lay within the 3′ untranslated region (3′UTR) (30). Transfection experiments revealed that deletion of the 3′UTR in DIO1 and GPX1 inhibited their Sec insertion activity. Interestingly, Sec incorporation in DIO1 was reconstituted with the 3′UTR of GPX1. The lack of conservation in primary sequence plus the formation of a stem-loop structure in the 3′UTR of DIO1 and GPX1 suggested that formation of the mRNA secondary structure is the main determinant for Sec incorporation activity and specificity. This eukaryotic stem-loop structure located at the 3′UTR of selenoproteins was named the Sec insertion sequence (SECIS) element (30). Two other factors were subsequently found to be required for Sec incorporation. First, an essential SECIS binding protein (SBP) was discovered in 2000 (64), and that same year saw the discovery of the mammalian counterpart to SelB, eukaryotic elongation factor for Sec (eEFSec) (65, 66). Importantly, it has recently been demonstrated that these factors are sufficient for Sec incorporation *in vitro* (N. Gupta, L. DeMong, S. Banda and P.R. Copeland, submitted for publication), suggesting that the roles of additional factors are regulatory in nature. In the following sections we will discuss in detail each factor involved in the Sec incorporation mechanism, with an emphasis on the eukaryotic system.

SECIS elements

Eukaryotic SECIS elements are approximately 100-nucleotide segments found exclusively in the 3′UTRs of all selenoprotein mRNAs (Figure 1). Despite an overall low level of sequence similarity, SECIS elements have two highly conserved regions: 1) the AUGA SECIS core and the apical AAR motif. The AAR (R=G or A) motif consists of a stretch of two adenines followed by A or G at the apical loop (67). The AUGA SECIS core is located at the base of the apical stem and is composed of a non-Watson-Crick G-A/A-G tandem pair structure with the AUGA sequence at the 5′ side and a GA pair at the 3′ side (68). The SECIS core is a hallmark of a broader group of RNA structures known as kink turns (k-turns) (69), which are found in rRNAs, snoRNAs, and some mRNAs. Both the AUGA and the AAR motifs were shown to be essential for Sec incorporation activity (67). SECIS elements can be divided into two groups according to their apical loop structure (70). The apical loop of form 1 is considered 'open', whereas form 2 has an internal bulge and a small stem-loop (71). The AAR motif is placed in the open loop of form 1 and within the internal bulge of form 2. Most human SECIS elements are form 2 with the exception of SelN, SelV, Dio1, GPX1, and GPX2 that are classified as form 1 (72). Functional relevance of form 1 vs. form 2 is not understood. The positioning of the SECIS element does not follow any stringent rules, except that it must be at least 51 nucleotides downstream of the UGA codon that encodes Sec (73). As illustrated in Figure 2, there is large variance in the distances between UGA codons and SECIS elements among the complete set of human selenoproteins. Selenoprotein O is at one extreme with only 104 nucleotides between the Sec codon and the SECIS element, and the type 2 deiodinase is at the other with a 5200-nucleotide spacing. The other striking feature about Sec codon positions is that they can be found anywhere in the open reading frame (see Figure 2). This is consistent with the fact that the placement of a SECIS element downstream of a reporter gene open reading frame with an in-frame UGA codon is sufficient to support Sec incorporation. However, this notion was recently challenged by the finding that in the ciliated protozoan, *Euplotes crassus*, UGA codes for both Cys and Sec in the same mRNA. So in the case of *Euplotes* TrxR 1 (TR1), only the UGA codons found in the 3′ end of the gene could encode Sec, and this was dependent on the presence of the correct SECIS element (74). Whether this phenomenon is confined to this particular species has yet to be determined.

Related to codon selectivity is the fact that only one of the 25 human selenoprotein mRNAs (SPS2) uses UGA as a stop codon, despite the fact that the SECIS element is well downstream of both UGA codons (75). Paradoxically, this

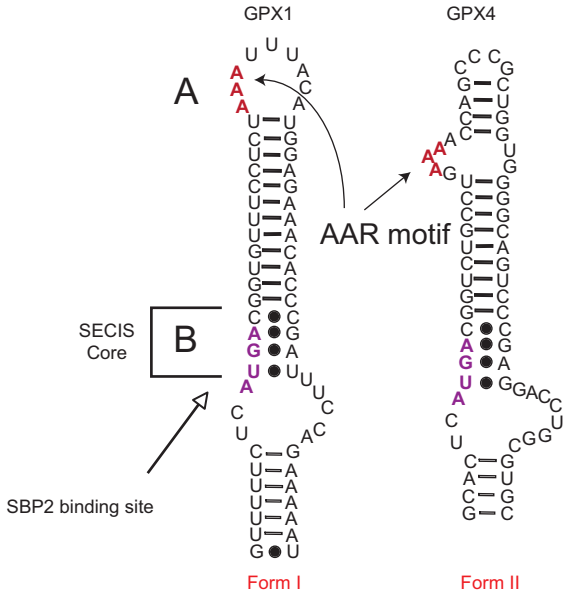

Figure 1 Predicted SECIS element structures for GPX1 (form I) and GPX4 (form II).
(A) The forms differ by the position of the conserved AAR motif (red), which is in the terminal loop for form I and in a 5′ bulge in form II.
(B) The conserved AUGA motif is shown in purple and represents the SECIS core, which refers to the region containing non-Watson-Crick base pairs. The basal stems (below the SECIS core) are not shown in their entirety and are of variable lengths.

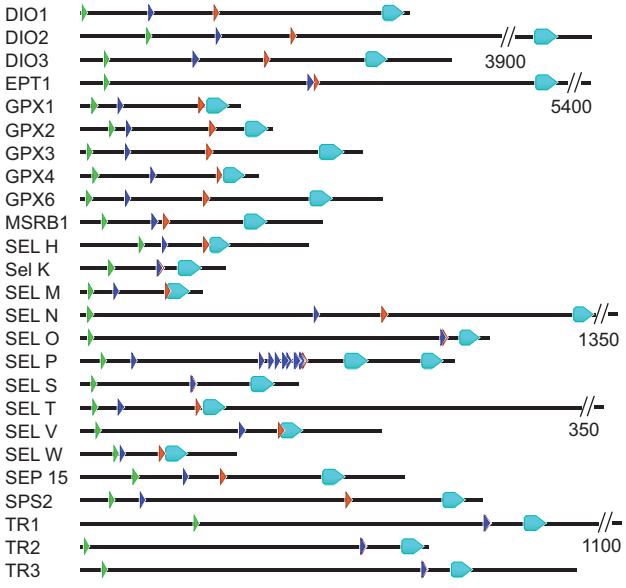

Figure 2 Relative distributions of start codons (green), Sec codons (blue), stop codons (red) and SECIS elements (light blue) within all 25 human selenoproteins.
All mRNAs are drawn to scale except for those with the indicated number of bases inserted between the slash marks.

finding supports the idea that Sec codons can exist anywhere (thus the lack of UGA termination codons in selenoprotein mRNAs) while also supporting the idea that not all eligible UGA codons will encode Sec. It is tempting to speculate that the UGA termination codon in SPS2 is somehow a selenium sensor, allowing Sec incorporation only when selenium concentrations are high, thus perhaps encoding a C-terminal peptide that creates a negative feedback loop by targeting the protein for degradation.

SECIS binding proteins

After the discovery of the SECIS element in 1991, several groups were simultaneously searching for an SBP with SelB-like functions (76–81). Many of the SBP candidates failed to display specificity for the apical loop or AUGA SECIS core. However, one among these was a 120-kDa protein from rat testis that was found to specifically interact with the AUGA SECIS core but not the apical loop region (78). The protein was subsequently purified and dubbed SPB2 because it was the second of the above-listed candidates to be reported (82). The purified protein was identified as an 846-amino-acid protein of unknown function, but the development of an *in vitro* Sec incorporation assay allowed the authors to demonstrate that SBP2 was essential for Sec incorporation (83). SBP2 consists of three distinct domains: an N-terminal domain (~400 amino acids) with no known function (and no similarity to other proteins with known functions), a central Sec incorporation domain (SID) that is also unique, and a C-terminal L7Ae RNA binding domain (RBD). A detailed treatment of SBP2 structure and function is presented in the next section.

A homologue of mammalian SBP2 was identified in BLAST searches, termed SBP2-like protein (SBP2L) (83, 84). Although the N-terminal portions of SBP2 and SBP2L are quite divergent, the C-terminal halves, possessing the Sec incorporation and RBDs, share about 45% amino acid identity (84). Functional characterization of SBP2L showed that it does specifically bind the AUGA core, albeit having generally weaker SECIS affinity than SBP2, with a few exceptions (85). Indeed, SBP2L was found to be stably associated with different SECIS elements *in vivo*, but despite the sequence homology with SBP2 and *bona fide* SECIS binding, mammalian SBP2L was unable to support (or inhibit) Sec incorporation *in vitro* (84–86). According to phylogenetic analysis, SBP2 and SBP2L are paralogs that were separated in a gene duplication event during early vertebrate evolution (84). Interestingly, many conserved regions of the invertebrate sequences are found in vertebrate SBP2L but not in SBP2, implying that invertebrate SBP2 is in fact more closely related to SBP2L than to SBP2. Strikingly, SBP2L from *Capitella capitata* (a polychaete worm) was competent for Sec incorporation *in vitro* (85), demonstrating that vertebrate SBP2L has lost its fundamental ability to support the Sec incorporation reaction. Further work in a mammalian system will be required to decipher the as yet mysterious function of SBP2L.

The ribosomal protein L30 (rpL30) has been identified as another SBP with specific AUGA SECIS core binding (87). Prior work has shown rpL30 to play a role in regulating its own splicing and translation (88) as well as in the induction of large-scale functional conformations in the translating ribosome (89). Like SBP2, L30 contains an L7Ae motif that is involved in the recognition of RNA k-turn structures (69). The rpL30 protein binds to k-turns of the 5′ splice site of rpL30 mRNA and helix 58 of the 28S ribosomal RNA (rRNA) (89, 90). Chavatte et al. (87) discovered rpL30 to have *in vitro* and *in vivo* interactions with different SECIS elements but not with the SECIS core deletion mutant. Mammalian cells that were transiently transfected cells with rpL30 showed a 2-fold stimulation for Sec-UGA read-through. Additional *in vitro* experiments confirmed that rpL30 can displace SBP2 from the SECIS by competing for the same binding region. The proposed model is that rpL30 could take part in Sec incorporation efficiency as a recycling factor for SBP2 (87).

Domain structure and function of SBP2

SBP2 is composed of three domains that were identified by their distinctive structural and functional features. A putative regulatory domain consists of the N-terminal half of SBP2, which is not required for Sec incorporation. This is underscored by the fact that the version of SBP2 found in invertebrates does not possess the N-terminal domain (84). Specifically, mammalian SBP2 without the N-terminal domain (rat CT-SBP2$^{399–846}$) and *Drosophila* SBP2 were both shown to be fully competent for Sec incorporation *in vitro* (86, 91). However, Papp and colleagues found evidence for an important role for the N-terminal half of SBP2 in live cells (92). In this study, it was experimentally confirmed that a predicted nuclear localization signal (NLS) was present within the N-terminal domain at residues 382–385 (83, 92). Nuclear localization was only observed when nuclear export was blocked by leptomycin B, consistent with prior work that showed exclusively cytoplasmic localization (93) and demonstrating that SBP2 can shuttle between the cytoplasm and the nucleus. This study went further to determine that under oxidizing

conditions, SBP2 becomes localized to the nucleus and selenoprotein synthesis is reduced (92). This seemingly paradoxical response to oxidative stress may only occur under extreme conditions where SBP2 would be inactivated by oxidation. This is consistent with the finding that oxidized SBP2 is unable to bind SECIS elements *in vitro* (82), suggesting that nuclear localization may be required to sequester and perhaps reduce oxidized SBP2 by the thioredoxin system (92). Although inhibiting Sec incorporation during oxidative stress is paradoxical, it may be a means to regulate selenium metabolism (i.e., reduce the demand for potentially toxic selenium intermediates like selenophosphate), which could exacerbate the oxidant stress. Whether nuclear localization is the only role for the N-terminal domain of SBP2 remains to be experimentally determined.

Truncation analysis of SBP2 led to the identification of two separate domains in the C-terminal half that together are essential for the four known SBP2 functions: Sec incorporation, SECIS interaction, ribosome binding, and eEFSec binding (86). These domains were termed the SID and the RBD. The intervening sequence between SID and RBD is not conserved and is not required for SBP2 function (94). The L7Ae RNA binding motif is located within the RBD, which contains several conserved features that are not typically found in L7Ae RBDs, but which are essential for SECIS binding (83, 84, 86). The core L7Ae motif is conserved in all known k-turn binding proteins and contains an invariant glycine residue that is critical for protein-RNA interactions. Substitution of G669 to arginine abolished SECIS interactions and function, thus validating the nature of the L7Ae motif in SBP2 (83). On its own, the RBD specifically interacts with SECIS elements with an apparent dissociation constant of ~400 nM, about four times higher a value than observed with intact SBP2. This has led some to consider the SID to be part of the RBD (94), but there is no evidence that the SID participates directly in RNA binding. Rather, it appears that the SID promotes a high-affinity conformation for the RBD (95).

In cell extracts, both endogenous SBP2 and exogenously added SBP2 are predominantly associated with ribosomes (86, 91–93). SBP2 can specifically interact with the 28S rRNA of 60S subunits and with fully assembled 80S ribosomes (86, 91). Interestingly, both the SID and the RBD were found to play a role in ribosome binding (86), and mutational analysis demonstrated that the SECIS binding and ribosome binding functions largely overlap. Consistent with this finding was the fact that SECIS elements were able to efficiently compete SBP2 off ribosomes, suggesting that simultaneous binding may not occur *in vivo* (96). These results suggested that the SBP2 ribosome interaction may function independently of SBP2 SECIS binding. Indeed, an analysis of ribosome conformation as a function of SBP2 binding indicates that the interaction may play a role in Sec-tRNA^{Sec} accommodation into the ribosomal A/P site rather than initial binding (97).

The SBP2 SID has been mutationally dissected into two regions, one contributing to high-affinity SECIS binding and the other providing an as yet undetermined role that is required for Sec incorporation, but none of the other known activities (95). Insight into the functional interplay between the SID and RBD was obtained when it was observed that the SID and RBD domains, when expressed as separate proteins, are fully active for SECIS binding and Sec incorporation *in vitro* (95). Co-immunoprecipitation (Co-IP) experiments with SECIS RNA, and recombinant SID and RBD proteins provided evidence for functional interdomain interactions in SBP2. Stable interactions between the SID and the RBD were dependent on the presence of wild-type SECIS RNA. Interestingly, a C-terminal mutant of SID (SID-IILKE$^{526\text{-}530}$) did not form a stable complex with the RBD-SECIS but still provided wild-type levels of SECIS binding by the RBD. From these results, a model of SBP2 binding the SECIS element was proposed: SBP2 initially interacts with the SECIS via the RBD that triggers a conformational change to recruit the SID. Subsequently, the SID-RBD interactions are likely stabilized by the IILKE$^{526–530}$ region (98). The current model for how SBP2 participates in the Sec incorporation reaction is discussed below.

A specialized translation elongation factor

During a typical elongation cycle of protein synthesis, the elongation factor eEF1A (EF-Tu in bacteria) delivers one of the 61 aa-tRNAs to the A site of the ribosome. The ribosome, being a complex ribozyme, then catalyzes peptide bond formation in the peptidyl transferase center (PTC), which transfers the nascent peptide from the P site tRNA onto the A site tRNA. Another elongation factor, eEF2, acts as a translocase and moves the peptidyl-tRNA into the P site, and the deacylated tRNA moves into the E site from where it is released. This frees the A site, and the ribosome is poised for the next elongation cycle [reviewed in (99)].

Sec incorporation can be thought of as a specialized elongation cycle, where a dedicated, Sec-specific elongation factor that binds only Sec-tRNA^{Sec}, replaces eEF1A. This G protein is present in both eukaryotes and prokaryotes as eEFSec and SelB, respectively, and is required for the co-translational incorporation of Sec (61, 65). However, because eEFSec specifically only delivers the Sec-tRNA^{Sec}

in response to an in-frame UGA codon that lies upstream of a SECIS element, ribosomal access is highly regulated. eEFSec has evolved structural elements that allow it to perform specialized functions that are not performed by eEF1A. In order to highlight the specialized functions of eEFSec, it is necessary to first review the functions of eEF1A during translation elongation.

eEF1A is one of the most abundant proteins in the cell (100). It has three distinct domains that perform specific functions. Domain I is also known as the G domain and is responsible for eEF1A's guanosine triphosphatase (GTPase) activity, which is stimulated by the ribosome. GTP-bound eEF1A has high affinity for aa-tRNAs, which are released when the GTP is hydrolyzed (101). Domain II functions in aa-tRNA binding, specifically the acceptor stem of the aa-tRNA, whereas domain III makes contacts with the T arm of aa-tRNA (102). Domains I and II are also involved in binding eEF1Bα, the guanine nucleotide exchange factor (GEF) for eEF1A (103). Structural studies indicate that domains II and III act as a single rigid unit during nucleotide exchange (102). Additionally, domain III is also required for the actin binding and bundling function that is specific to eEF1A, and is absent in EF-Tu (104). Therefore, a combination of structural, kinetic and genetic studies have given detailed insight into the canonical function of eEF1A in translation and can be used as an important reference point in assessing the role of eEFSec in Sec incorporation.

Eukaryotic EFSec and eEF1A are highly divergent with only ~35% pairwise intraspecies identity from *Drosophila* to humans. Despite these differences, there is significant structural similarity in the domain structure of eEF1A and eEFSec (105, 106). The major exception is a C-terminal extension (domain IV) in eEFSec, which is absent in eEF1A. The same is true for the prokaryotic version of eEFSec, SelB, although the fourth domain in SelB is not evolutionarily related to that in eEFSec. This high degree of diversity is undoubtedly related to the specialized nature of eEFSec and SelB, and in that context, the following sections describe the progress that has been made in deciphering the mechanism of eEFSec and SelB specificity.

Nucleotide hydrolysis and exchange

eEF1A and eEF2 are G proteins that use GTP hydrolysis as an allosteric effector to facilitate aa-tRNA delivery into the A site of the ribosome and catalyze translocation (107). Specifically, GTP hydrolysis plays a critical role in conformational changes of eEF1A and eEF2 during translation elongation. The intrinsic GTPase activity of translation elongation factors is low and is stimulated by the ribosome by several orders of magnitude (108). GEFs are utilized to exchange the GDP for GTP after hydrolysis and return the elongation factor to its 'active conformation' for tRNA binding. Similar to canonical elongation factors, SelB utilizes GTP binding and hydrolysis for Sec-tRNA^{Sec} specificity and delivery to the ribosomal A site (109). Detailed analysis of SelB kinetics has shown that SelB bound by GTP has more than a millionfold higher affinity for Sec-tRNA^{Sec} than its GDP-bound or apo form (110). Moreover, this thermodynamic coupling between Sec-tRNA^{Sec} and GTP binding to SelB is not observed with Ser-tRNA^{Sec} or deacylated tRNA^{Sec}. Thus, there is a stabilization of the SelB/GTP/Sec-tRNA^{Sec} ternary complex in a specific manner. Additionally, a recent study has shown that there is substantial conformation change in SelB upon binding GTP (111). Together, these studies argue for a model where rapid release of Sec-tRNA^{Sec} from SelB, and into the ribosomal A site, occurs upon GTP hydrolysis on the ribosome. Both eEFSec and SelB bind to GTP with significantly higher affinity than GDP, thus suggesting that, unlike eEF1A, these factors do not require a GEF to function (65). This raises questions about the mechanism and purpose of nucleotide exchange during the ribosomal delivery of Sec-tRNA^{Sec}, although it may simply support the idea that there is no physiological role for eEFSec:GDP, as opposed to eEF1A:GDP, which is proposed to interact with actin (112). Interestingly however, an effort to identify eEFSec binding partners in *D. melanogaster* has led to the discovery of a protein termed dGAPSec (113). dGAPSec is a GTPase-activating protein identified in this study using a yeast two-hybrid system with *Drosophila* eEFSec as the bait. It was shown to support SECIS-dependent UGA read-through but was not required for endogenous selenoprotein biosynthesis (113). The specific contribution of dGAPSec to Sec incorporation remains to be assessed.

Domain IV

The feature that most distinguishes eukaryotic and prokaryotic Sec incorporation is that the former requires SBP2. The experimental evidence to date supports the idea that the SBP2/SECIS complex recruits eEFSec ternary complex to the ribosome for Sec-tRNA^{Sec} delivery. The interaction between SBP2 and eEFSec was first reported as an RNAse-sensitive interaction when both factors were transiently transfected (65). Co-IP has generally not been successful, but an electrophoretic mobility shift assay has permitted the presumably transient

interaction to be captured. Using this assay, recent work has shown that eEFSec is able to interact with SBP2 in a SECIS-dependent but Sec-tRNA^{Sec}-independent fashion (95, 114). Importantly, conserved sequences in the C-terminal portion of domain IV were found to be required for both tRNA and SBP2 binding, whereas sequences at the N-terminal region of domain IV were either dispensable or played a role in tRNA binding but not SBP2 binding (114). Although the involvement of domain IV in SBP2 binding was expected, its role in tRNA binding is surprising, and the extent to which it plays a regulatory role in Sec-tRNA^{Sec} binding will require further investigation. In addition to SBP2 and tRNA binding, there is also evidence that domain IV may be involved in regulating GTP hydrolysis (114). Mutation of a conserved region in this domain showed increased intrinsic GTP hydrolysis compared to wild-type eEFSec (114). Thus, there is now additional evidence to argue for a conformational change induced by GTP hydrolysis that involves domain IV, and structural and/or biophysical studies of eukaryotic eEFSec are needed to confirm this. All together, the recent results clearly indicate that eEFSec domain IV is pleiotropic and is likely a key player in the conformational dynamics that are required for Sec incorporation.

A mechanistic model for Sec incorporation

The fundamental question about the mechanism of Sec incorporation is one of specificity. The cell contains an eEFSec ternary complex that should be able to act as a UGA suppressor, but this occurs only in the presence of an SBP2/SECIS complex. The crux of the question, therefore, probably lies in the mechanism by which eEFSec gains access to the ribosomal A site. Secondarily, the question of how the Sec-tRNA^{Sec} is accommodated and whether GTP hydrolysis plays the same role for eEFSec as it does for eEF1A must also be considered.

Based on currently available data and a healthy dose of speculation, we propose the following sequence of events. SBP2-bound ribosomes specifically recruit SECIS-containing mRNAs, and the subsequent SBP2/SECIS complex recruits eEFSec ternary complex. This mechanism invokes a SECIS-directed translation initiation event, thus providing a potential basis for requiring a SECIS element in *cis*. Once the ribosome reaches the Sec codon, the SBP2/SECIS/eEFSec complex is able to access the A site and deliver the Sec-tRNA^{Sec}. Upon tRNA binding, GTP hydrolysis ensues and eEFSec is released. During the process of tRNA accommodation, we speculate that the SECIS adopts a conformation that is unfavorable to SBP2 binding, allowing it to rebind the ribosome in order to conformationally facilitate Sec-tRNA^{Sec} movement through P and E sites. This final step also serves to complete the cycle, allowing SBP2 to recruit another selenoprotein mRNA. The involvement of translation initiation is somewhat confounded by the observation that initiation factors are not required for Sec incorporation (115), but it is possible that all of this is occurring at the level of the ribosomal subunits, thus bypassing the canonical initiation process. Although largely untested, this model can provide the framework for the current efforts to fully decipher the mechanism of eukaryotic Sec incorporation.

Regulation of selenoprotein synthesis

mRNA decay and Sec insertion inhibition by a novel SBP

Previous experiments in rats found a direct correlation between dietary selenium levels and GPX1 mRNA and protein expression. Selenium-deficient rats had an ~90% decrease in GPX1 mRNA levels, whereas GPX4 mRNA levels remained constant (116). Moriarty et al. proposed that nonsense-mediated decay (NMD) could be involved in the degradation of GPX1 mRNA (117). NMD is an mRNA surveillance mechanism that detects premature stop codons and avoids the translation of nonfunctional truncated proteins by promoting rapid mRNA degradation [reviewed in (118)]. Normal mRNAs have their stop codon downstream of the final intron/exon boundary, but mRNAs with a premature stop codon will have a terminating ribosome upstream of the final exon junction. Through the action of factors bound to the exon junction, a translation termination event that occurs upstream of the final exon provides a signal to ribosome-associated NMD factors that promote mRNA degradation. Interestingly, most Sec codons are upstream of the final exons, but only a few mRNAs, e.g., GPX1, have been demonstrated to be susceptible to degradation, and only when selenium levels are limiting (119, 120). Specifically, when the Sec codon was moved downstream of the final exon junction, GPX1 mRNA levels remained constant regardless of selenium status (121, 122). Interestingly, GPX1 expression has also been reported to be regulated at the translational level, suggesting a dual mechanism for regulation (49, 119). The role of the SECIS element in regulating mRNA stability is not clear, as neither the GPX1 nor GPX4

SECIS element has been demonstrated to be both necessary and sufficient for regulation (119, 123).

In addition to regulation at the level of mRNA stability, there is recent evidence for translational regulation as well where a potential role for the SECIS element was found to reside in a novel SBP, eIF4A3 (49). A component of the exon-junction complex that marks intron/exon boundaries, the eukaryotic initiation factor 4A3 (eIF4A3), was found in UV-cross-linking experiments as a 48-kDa band that bound specifically to the GPX1 SECIS probe (49). Interestingly, the addition of recombinant eIF4A3 to an *in vitro* Sec incorporation assay reduced the expression of a luciferase reporter with the GPX1 SECIS element, whereas the activity of a luciferase/GPX4 reporter was unaffected. To elucidate the biological relevance of eIF4A3 in GPX1 regulation, the effects on mRNA and protein levels were measured from mammalian cells in different selenium conditions. Protein levels of eIF4A3 increased by ~2.5-fold, whereas GPX1 decreased by ~3-fold in selenium-deficient cells compared to selenium-supplemented cells. GPX1 protein levels were back to normal when eIF4A3 was knocked down in selenium-deficient cells. Overexpression of eIF4A3 protein reduced GPX1 protein levels even in cells supplemented with selenium. In addition, more ribonucleoprotein (RNP) complexes of GPX1 mRNA with eIF4A3 were formed under low-selenium status. This set of experiments served as clear evidence that selenium-dependent overexpression of eIF4A3 and the subsequent association of eIF4A3-GPX1 RNP can indeed regulate GPX1 expression *in vivo*. Thus, it is emerging that both stability and translation of selenoprotein mRNAs may be highly regulated at least in part by SBPs that could regulate SBP2 access.

Selenoprotein P

One selenoprotein more than any other challenges all of the mechanistic assertions made above. SelP is a selenoglycoprotein that is synthesized in the liver and secreted into the plasma, accounting for ~70% of plasma selenium (124–126). What makes it unique among selenoproteins is the fact that it contains 10 Sec codons upstream of a highly conserved 3′UTR containing two SECIS elements. Identified in 1982 (124), early studies demonstrated that tryptic peptides of SelP contained selenium at multiple sites within the protein, thus showing that SelP is a selenoprotein with more than one Sec residue (127). Interestingly, a single Sec residue resides in the N terminus, whereas the remaining are primarily located in the C terminus (128). The N terminus of SelP has been shown to have a potential weak antioxidant role, whereas the C terminus is essential for selenium distribution (64, 129). Knockout of the gene encoding SelP (*SEPP1*) has implicated SelP in being primarily responsible for delivering selenium to the brain and testes (130, 131).

SelP mRNA translation

SelP is a conundrum of the translational coding machinery. The central question from a mechanistic standpoint is how the Sec incorporation process, which has been generally described as quite inefficient (see below), is able to accommodate efficient SelP production. In an early analysis of SelP mRNA sequence, conserved regions were found that could potentially regulate SelP incorporation (132). The 5′UTR of rat SelP was initially shown to be short (approximately 38 bases) and not very conserved, but a recent study has shown that human SelP exists as three alternative transcripts differing only in their 5′UTR lengths (133). The smallest 5′UTR (approximately 76 bases) corresponds to the most abundant transcript and also has a target site for micro-RNA mir7, which may potentially play a role in SelP translation efficiency. The 5′UTR of the three transcripts were predicted to fold with varying secondary-structure complexity, and it was speculated that this variation could in turn alter the rate and efficiency of SelP translation (133).

The SelP coding region, which is approximately 1000 bases, has been reported to have five conserved regions in the range of 81–100% in humans and rat mRNA, and within these conserved regions are located at least six of the UGA codons (132). This very high degree of sequence conservation is probably maintained for structural and functional regulation. However, the possibility of these conserved coding region sequences functioning as cis-acting elements has never been explored. Regions of conservation in the coding region lend support to the idea that a regulatory structural element may play a role in Sec incorporation efficiency for some selenoproteins, including SelP. This element, termed the Sec recoding element, was shown to regulate the efficiency of read-through in the absence of a SECIS element (134, 135), suggesting that its function may be mechanistically distinct from the core Sec incorporation reaction.

Perhaps the most important feature in SelP mRNA is the approximately 800-base-long 3′UTR, which also been found to have a high degree of sequence conservation (132). Given the length of the SelP 3′UTR and its highly conserved sequences, it appears that the non-SECIS portions of the SelP 3′UTR may be crucial for regulating incorporation of Sec. Indeed, the deletion of the first 83 nucleotides of the

SelP 3′UTR increases translation of a luciferase reporter construct with a single UGA codon (M. Gupta, S. Shetty, and P.R. Copeland, unpublished data).

SelP SECIS

The fact that the SelP mRNA possesses two SECIS elements has made them a target of investigation as to whether incorporation of multiple Sec requires the presence of more than one SECIS and also whether the two SECIS elements vary in their ability to incorporate Sec. In the first such study, SelP SECIS elements were analyzed for their ability to support Sec incorporation into the DIO1 coding region (67). It was found that the upstream SelP SECIS 1 was three times more active than that of DIO1. The downstream SelP SECIS 2, on the other hand, was less efficient, having almost the same activity as the DIO1 SECIS. This was the first evidence that the two SECIS elements vary in their ability to incorporate Sec and possibly play different roles in SelP production. A study by the Howard group showed that in rabbit reticulocyte lysate, SECIS 1 alone can incorporate two Sec residues in a reporter construct, whereas SECIS 2 did so with much lower efficiency, once again proving that SECIS 1 is more efficient than SECIS 2 (136).

Transfection of a GST-SelP fusion cDNA showed that even with deletion of SECIS 2, a full-length product was obtained. In contrast, the deletion of SECIS 1 failed to support any production of full-length protein, strongly supporting the theory that SECIS 1 plays a crucial role in full-length SelP synthesis (137). SelP SECIS elements have also been analyzed for their individual binding affinity for SBP2 (K_d values are 1.64 nM for SECIS 1 and 3.4 nM for SECIS 2) and are not very far apart (85). Overall, these combined studies suggest that the SelP 3′UTR likely assembles a unique set of factors that are required to achieve efficient and processive Sec incorporation *in vivo*.

Current models for Sec incorporation within SelP: reflections on Sec incorporation efficiency

The main question derived from a consideration of how SelP is synthesized is one of efficiency. Considering the complexity of regulatory events that take place at the SECIS element, the efficiency of the Sec incorporation reaction has been a topic of interest in the past decade. The dual function of the UGA codon as a stop signal and Sec insertion indicates that there is competition between these two events. Suppmann et al. published the first article that addressed the question regarding bacterial Sec incorporation (138). Using a bicistronic reporter system where the Sec codon is placed between two open reading frames, they found that Sec incorporation (production of the downstream protein) occurred with ~5% efficiency. Overexpression of the Sec incorporation machinery (SelB, Sec-tRNA^{Sec}, and SecS) increased efficiency to a maximum of 10%. The authors concluded that the inefficiency of bacterial Sec incorporation is caused by the slow insertion rate of SelB/GTP/Sec-tRNA^{Sec} competing with UGA termination through release factor RF2 (138), but direct testing of this hypothesis has not been reported.

Mammalian Sec incorporation efficiency was also determined by reporter constructs measuring the ratio of translation termination products to Sec incorporation both *in vitro* and in transfected cells (93). Sec incorporation activity was highly inefficient in both cases with only 5–8% efficiency *in vitro* and <1% efficiency in transfected rat hepatoma cells (McArdle 7777). In contrast, several endogenous selenoproteins are found to be highly expressed in mammalian tissues. For example, testicular GPX4 (139), plasma SelP (127), and the ubiquitous TrxR1 (140) are produced in high quantities. In fact, the calculated Sec incorporation efficiency for endogenous TrxR1 is 87% in human blood cells (141). Indeed, more studies are needed to clarify the discrepancy between the tissue samples and the *in vitro* systems. One explanation could be that bacterial Sec incorporation is intrinsically inefficient, whereas mammalian organisms might have several efficiency factors expressed in a tissue-specific and *in vivo*-dependent manner.

The *in vivo* efficiency of SelP synthesis is likely to be quite high. As an abundant plasma protein, large amounts of the protein can be purified from fresh plasma with a concentration estimated to be 26 μg/ml of plasma (127, 142), and a prominent single band of the expected molecular weight is observed when human hepatoma cells are labeled with ^{75}Se in cell culture (143). When SelP is purified from plasma, minor species corresponding to premature termination events at upstream Sec codons are recovered (144), suggesting that Sec incorporation into SelP is not an 'all-or-nothing' event. The translation of SelP mRNA *in vitro* has been reported to result in full-length protein, but with the prominent production of premature termination products (93).

In an effort to gain more insight into the efficiency process, polysome loading studies were conducted on transfected SelP mRNAs and endogenous SelP mRNAs expressed in human kidney (HEK293) cells (65). It was found that both the transfected and endogenous mRNA

had the same number of ribosomes loaded. However, a study comparing ribosomal loading on selenoprotein vs. non-selenoprotein mRNA showed that a larger number of ribosomes were associated with non-selenoprotein mRNA, suggesting inherently lower translation elongation rates for selenoprotein mRNAs (145, 146). Because lower rates do not necessarily correlate with premature termination, the sum of evidence suggests that SelP production *in vivo* is efficient and that *in vitro* systems are likely lacking trans-acting factors that may be required specifically for SelP production.

Fixsen and Howard have recently uncovered a processive mechanism in Sec insertion that might explain SelP *in vivo* synthesis (136). Sec incorporation activity was measured by *in vitro* translation with a dual luciferase reporter gene having a SelP 3′UTR and either one UGA or multiple UGA codons. As expected, the efficiency of the first Sec-UGA codon was ~10%, but surprisingly, incorporation at all of the subsequent downstream UGA codons were highly efficient at ~68–87% (136). From these results, suggesting that a small number of ribosomes (~10%) that are competent for Sec insertion pass the first Sec-UGA and continue to be 'primed' for downstream Sec-UGA codons. These Sec-competent ribosomes can be 'diluted' under high amounts of selenoprotein mRNA but can be 'enriched' to selenoprotein mRNA by displacing non-Sec-competent ribosomes to non-selenoprotein mRNA. Recent data argue in favor of the existence of heterogeneous populations of eukaryotic ribosomes [reviewed in (147)]. Under different conditions, ribosomes with a distinctive set of ribosomal proteins could participate in mRNA-specific translation events. For example: depletion of Rps25, a nonessential ribosomal protein, retained normal levels of total translation but specifically decreased viral internal ribosome entry site (IRES) activity in cells by inhibiting 40S subunit association with the IRES structure (147). However, the hypothesis of functionally different ribosomes for certain cellular mRNAs faces the challenge of specificity. As described above, it is possible that the SBP2/SECIS complex is involved in specifically recruiting primed ribosomes, and in the case of SelP, it is likely that other 3′UTR binding proteins may participate in creating a ribosome that can processively incorporate Sec.

Another model for SelP translation posits that the circularization of the SelP mRNA by interaction of the polyA tail and translation initiation factors positions SECIS 2 on the first UGA and SECIS 1 on the subsequent UGA codons. The first UGA is primarily decoded by SECIS 2, which has been shown to be inefficient, and this leads to high termination product at this UGA. The more efficient SECIS 1, however, is responsible for the processivity and incorporation of Sec downstream. Slow incorporation at the first UGA by SECIS 2 leads to ribosomal pausing and may be the reason why fewer ribosomes are associated with SelP mRNA (137).

Although it is not possible to distinguish between these two models at this time, all current data indicate the following: 1) Incorporation at the first UGA is most inefficient. 2) SECIS 1 is more efficient than SECIS 2. 3) SelP synthesis *in vivo* appears to be more efficient than *in vitro* or in transfections. 4) A larger UGA codon context may modulate efficiency. 5) Conserved regions within the coding and 3′UTR may play regulatory roles. 6) SECIS 1 may be more efficient than SECIS 2, but the severalfold higher Sec incorporation observed for DIO1 with the full length SelP 3′UTR than with its own 3′UTR supports the recruitment of other trans factors by SelP 3′UTR. This is further supported by the fact that reporter construct of GST-SelP with only SECIS 1 yielded full-length product, albeit with lower efficiency, thus supporting the theory that the downstream conserved sequence and SECIS 2 of SelP 3′UTR are essential for efficiency. Furthermore, from the fact that transfected plasmids do not lead to enhanced expression, it seems more likely that besides the cis elements located in the SelP mRNA, other trans factors may play a role in SelP translation.

More than two decades have passed since SelP was identified, yet several outstanding questions still remain on its synthesis, regulation, and processivity. Indeed, the synthesis of SelP stands as one of the most challenging problems associated with the mechanism of Sec incorporation. As such, deciphering this mechanism will undoubtedly shed light on a multitude of regulatory networks that control not only SelP synthesis but general features of mRNA translation and stability as well.

Summary and conclusion

In the past three decades, tremendous progress has been made in identifying the factors that are required for and involved in the complex process of Sec incorporation. Moving forward, the goals should clearly move toward understanding how these factors work in concert to achieve the critical balance of selenium metabolism and the regulated expression of selenoprotein production in the context of varied oxidative stress. A complete understanding of the molecular biology of Sec incorporation should allow the development of clinically useful small molecules that can modulate selenoprotein production,

maximizing their beneficial aspects without the burden of selenium supplementation.

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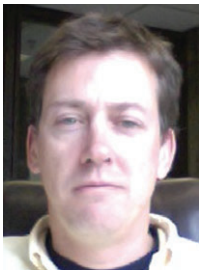
Jonathan N. Gonzalez-Flores, PhD, is currently a postdoctoral fellow at the Ponce School of Medicine and Health Sciences. He did his PhD research training in Biochemistry and Molecular Biology under the guidance of Dr. Paul R. Copeland at Robert Wood Johnson Medical School. He received a Bachelor of Science degree in biology from the University of Puerto Rico-Mayagüez Campus. Dr. Gonzalez has been supported by multiple research training fellowships such as NSF-IGERT, NIH-Biotechnology Fellowship, and MARC undergraduate fellowship.

Sumangala Shetty, PhD focused her doctoral studies at Dusquesne University under the guidance of Dr. Mihaela-Rita Mihailescu on investigating essential long range RNA-RNA interactions in Hepatitis C virus (HCV) RNA. Dr. Shetty's current postdoctoral studies in Dr. Paul Copeland's lab at Robert Wood Johnson Medical School is centered on identifying novel RNA-protein interactions required for Selenoprotein P synthesis.

Aditi Dubey is a graduate student in Dr. Paul Copeland's lab, pursuing a PhD in Cell and Developmental Biology. Her research involves investigating the process of Selenocysteine (Sec) incorporation into proteins. Specifically, her focus is on the mechanism by which the selenocystyl-specific elongation factor, eEFSec, functions in this process. Prior to her graduate studies, Ms. Dubey received her Bachelors of Science in Biological Sciences in 2009 at Drexel University in Philadelphia, PA. At Drexel, Aditi worked in Dr. Aleister Saunders' lab alongside a graduate student to investigate the molecular mechanisms underlying the pathology of Alzheimer's disease.



Paul R. Copeland, PhD is an Associate Professor of Biochemistry and Molecular Biology at Robert Wood Johnson Medical School in Piscataway, New Jersey. Having received his doctoral degree from the University of Virginia in 1997 in the field of translational control under the guidance of Mike Wormington, Dr. Copeland went on to perform postdoctoral work in the laboratory of Dr. Donna Driscoll at the Cleveland Clinic Foundation. It was in Dr. Driscoll's lab that one of the key players required for Sec incorporation was discovered (SECIS binding protein 2). Dr. Copeland has pursued the mechanistic aspects of Sec incorporation in his own lab since 2002.