

# Chapter 2

## Eukaryotic Mechanisms of Selenocysteine Incorporation and Its Reconstitution In Vitro

Mark H. Pinkerton and Paul R. Copeland

**Abstract** Selenocysteine (Sec) incorporation requires the highly choreographed interplay between a multitude of factors and the elongating eukaryotic ribosome. This chapter focuses on the mechanism by which the known factors deliver Sec-tRNA<sup>[Ser]Sec</sup> to the ribosomal A-site for both the single Sec incorporation events required for most selenoproteins as well as multiple Sec incorporation events required for the synthesis of Selenoprotein P. The role that in vitro translation systems has played in the determination of these mechanisms is highlighted.

**Keywords** Ribosome • Rabbit reticulocyte lysate SECIS • SECIS binding protein 2 • Sec-specific elongation factor • Selenocysteine incorporation • Selenoprotein P • Translation • Wheat germ lysate

### 2.1 Introduction

Selenium (Se) is a trace element found and utilized in organisms across all domains of life and is an essential micronutrient for humans. Se makes its way into proteins via the amino acid selenocysteine (Sec), which was discovered as the twenty-first amino acid in the late 1970s [1, 2]. Sec is structurally similar to the amino acids serine (Ser) and cysteine (Cys) and is synthesized from a Ser precursor as described in Chap. 4. The mechanism of Sec incorporation into proteins is a requirement for many essential functions in humans. The importance of Se is observed as Se deficiency being the underlying cause of numerous diseases in humans. Selenoproteins have a higher enzymatic efficiency and faster chemical reaction rates with electrophiles than their Cys counter parts, which gives selenoproteins high redox potentials [3, 4]. In addition, enzymes harboring Sec instead of Cys in their active sites are much more resistant to oxidative inhibition [5]. Due to these redox characteristics,

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it is not surprising that Sec is found in antioxidant enzymes such as glutathione peroxidases, thioredoxin reductases, and iodothyronine deiodinases in humans and other eukaryotes. However, the importance of Sec and Se as a nutritional requirement varies among species in eukaryotes. For example, fungi and higher plants do not utilize Sec and completely lack the capacity for Sec incorporation. Other organisms, notably *Drosophila*, have the required machinery but synthesize only three selenoproteins that are not essential for survival, fertility or protection from oxidative stress [6].

The molecular machinery for Sec incorporation in eubacteria is well characterized in both synthesis and translation (see Chaps. 3–5), but only in the past two decades has the eukaryotic mechanism of Sec incorporation been deciphered. Sec incorporation in eukaryotes is dependent on *cis* acting factors within the mRNA and *trans* acting factors involved in bringing a selenocysteyl-tRNA (Sec-tRNA<sup>[Ser]Sec</sup>) to the ribosome. Sec-tRNA<sup>[Ser]Sec</sup> recognizes the UGA stop codon in selenoprotein mRNA, thus representing one of the exceptions to the canonical genetic code. Specifically, recoding will occur when a selenoprotein mRNA contains a stem-loop structure in the 3' untranslated region (3' UTR). This element was originally discovered to be required for the synthesis of type 1 iodothyronine deiodinase (DIO1) and was named the Sec insertion sequence element or SECIS element [7]. In a search for SECIS binding proteins, a 120 kDa factor was found to specifically interact with the *Gpx4* SECIS element [8]. This factor, eventually named SECIS binding protein 2 (SBP2 or SECISBP2) was shown to be required for Sec incorporation in vitro [9]. Soon after the discovery of SBP2, a Sec-specific elongation factor (eEFSec) was also found to be a specific Sec-tRNA<sup>[Ser]Sec</sup> binding factor [10, 11]. Recently, these core factors were shown to be the minimum requirements for Sec incorporation into proteins in a reconstituted cell free in vitro system [2]. There are, however, still underlying fundamental questions that remain about factors that may govern the efficiency and processivity of Sec incorporation. Furthermore, other factors are involved in regulating selenoprotein expression, which will be discussed later in this chapter. Here we review the current state of knowledge regarding the eukaryotic Sec incorporation machinery and discuss in vitro systems and their importance in furthering our understanding of selenoprotein expression.

## 2.2 UGA Recoding

Recoding is a translational event where the ribosome incorporates an amino acid different than that specified by the cognate codon and the canonical genetic code. This process fundamentally describes the mechanism by which Sec is incorporated. The programmed recoding of codons for different amino acids was once unknown in biology, but studies have discovered numerous ways organisms have altered the canonical genetic code for translation. UAA, UAG, or UGA are the standard termination codons for ribosomes, but only UAG and UGA appear to be the codons that are recoded in organisms [12]. The UGA stop codon appears to be the most

commonly recoded in *Mycoplasma* and mitochondria, both of which use UGA for tryptophan rather than to terminate translation [13, 14], while pyrrolysine is coded by UAG primarily in methanogenic archaea [15]. Typically, stop codon redefinition results from a difference of tRNA specificity, a lack of stop codon recognition by termination factors [16], or suppression due to lack of tRNA-Trp in *E. coli* [17]. In Sec incorporation, however, tRNA specificity and lack of UGA recognition does not play a part, instead it requires the combination of specific factors to work in harmony with the canonical translation elongation system to recode UGA for Sec. As such, this system is unique across all domains of life in the recoding of stop codons [18, 19].

## 2.3 SECIS

The translational machinery for Sec incorporation requires a mechanism to ensure that only selenoprotein mRNAs have their UGA codons recoded. Selenoprotein mRNAs contain the SECIS *cis* element in the 3' UTR, which is the only required *cis* acting element for Sec incorporation. SECIS was first reported after sequence alignments of type I iodothyronine deiodinase (*DIO1*) located in the 3' UTRs containing a loosely conserved sequence that was required for Sec incorporation both in vitro and in *Xenopus* oocytes [7]. It was elaborated further in follow up studies that SECIS elements are found exclusively the 3' UTR of mRNAs encoding Sec containing proteins [20].

SECIS elements contain highly variable sequences across species and different mRNA 3' UTRs, but share a similar secondary structure consisting of two helices, an internal loop, and an apical loop. The helices and loops of the SECIS elements are used to define the two forms of SECIS elements that occur. Form 1 contains a relatively large apical loop and a single internal loop separated by a 12–14 base-pair (bp) helix, while Form 2 has another short 2–7 bp helix, an additional internal bulge, and a smaller apical loop [21]. The apical loop contains AAR residues conserved in the apical loop, and the internal loop contains a conserved SECIS core consisting of an unpaired AUGA and UGR along the 5' and 3' sides. Both the apical loop and the internal loop are essential for Sec incorporation [20]. The AAR residue is required for Sec incorporation, but its function has not been elucidated and is further confounded by the SECIS elements found in SelM and SelO that contain CCX residues in place of the AAR [22, 23].

SECIS elements contain a conserved feature that defines their structure and function. The AUGA sequence of the SECIS forms non-Watson-Crick base pairs with a 3' side, which form a kink turn (K-turn) motif that generates an approximate 120° bend in the helix [24]. K-turn motifs are bound by proteins like SBP2 via the K-turn binding motif designated the L7Ae domain [25–29]. The functionality of K-turns may stem from their flexibility in conformation as they appear to move like a hinge to support variable conformations [30]. In the presence of  $Mg^{2+}$ , the hinge is found to be in its fully bent confirmation, which can explain the phenomenon of  $Mg^{2+}$

inhibition of SECIS/SBP2 binding [31]. The importance of the AUGA region is emphasized in a study of a single homozygous point mutation of the AUGA to ACGA in the SECIS element of selenoprotein N (*SEPNI*) in vivo, which causes congenital myopathies because the mutation prevents SBP2 binding to the SECIS element [32].

The SECIS element also has a hard coded ability to regulate Sec incorporation. The regulation is observed by large differences in UGA recoding efficiency both in vitro and in vivo of various SECIS elements in chimeric constructs [2, 33, 34]. Some of the predictors of increased translation efficiency included the presence of a GC base pair in helix 2 of the SECIS element and a U in the 5'-side of the internal loop [33]. SECIS elements also vary in their distance from the UGA, 104-5200 nucleotides away in known transcripts, but there appears to be a minimal spacing requirement of 51 nucleotides downstream of UGA [35]. In addition, the affinity of SBP2 for the SECIS element does not correlate with more efficient translation [33, 34], nor does the concentration of EFSec or SBP2 [36]. Currently, the mechanism by which SECIS elements regulate Sec incorporation efficiency remains a mystery.

## 2.4 SECIS Binding Protein 2

SECIS elements are made functional, at least in part, by the binding of SBP2, which was originally identified as a 120 kDa protein that specifically cross-linked to the *Gpx4* SECIS element [8]. Subsequent purification and characterization led to the discovery that it is essential for Sec incorporation [9]. The known functions of SBP2 include SECIS binding, ribosome binding and transient interaction with eEFSec. Truncation and site-specific mutagenesis of SBP2 was initially used to demonstrate that SBP2 contains three distinct domains: (1) an N-terminal domain of ~400 amino acids with no known function, a central “Sec incorporation domain (SID)” of about 100 amino acids; and (2) a C-terminal ~300 amino acid domain that contains the conserved L7Ae RNA binding domain [9, 25, 29, 37–39].

Interestingly, the SID and RNA binding domains are sufficient for all known functions of SBP2, thus demonstrating that the SBP2 N-terminal domain is not essential for Sec incorporation in vitro [9, 40]. Evidence for a function for the N-terminal domain comes from the fact that it contains discrete conserved stretches of amino acid sequence [41], and mutations in humans that are predicted to truncate the N-terminal domain (reviewed in [42]). In addition, the N-terminal domain is predicted to contain a lysine-rich nuclear localization sequence (NLS) [9]. The presence of a functional NLS was confirmed in a study of liver cells under conditions of high oxidative stress and when nuclear export was blocked by leptomycin B resulted in reduced selenoprotein levels. The reduction of Sec incorporation suggested that SBP2 may get shunted to the nucleus during oxidative stress perhaps to avoid oxidative damage [43].

The functional relationship between the SID and RNA binding domains is complex. Even when the SID and RNA binding domains are expressed separately as

individual proteins, they form a stable SECIS-dependent complex and retain all of their functions in Sec incorporation in vitro with the exception of stable ribosomal binding [39]. SID has been described as an extension of the RNA binding domain [44], but it does not make contact with the SECIS element and instead plays a role in increasing SECIS binding affinity of the RNA binding domain [39]. The complexity of the SID/RNA binding domain interplay is well illustrated by the fact that mutation of the conserved IILKE<sup>526–530</sup> to alanine eliminated Sec incorporation and stable interaction with the RNA binding domain, but it did not affect high affinity SECIS binding [39]. This same mutation in the intact C-terminal half of SBP2 results in complete inactivation of SECIS binding. From this, the authors concluded that physical linkage between the SID and RNA binding domain constrains conformational options, but the structural significance of this awaits high resolution structure determination.

As alluded to above, one role of SBP2 that is not well understood is the ability to bind to the ribosome. Recent work that followed the initial characterization of the interaction between SBP2 and the large ribosomal subunit (see review in [45]) included mapping ribosome conformational changes by selective 2'-hydroxyl acylation analyzed by primer extension [46] and mapping of the ribosomal binding sites of SBP2 on 28S rRNA to an expansion segment 7 L [47]. As tantalizing as these data have been, little is known about the actual role that SBP2 plays when stably bound to the ribosome. Resolution of this mechanism will require a substantial effort integrated with structural studies.

## 2.5 Sec-Specific Elongation Factor

eEFSec is central to the Sec incorporation process as the factor that delivers Sec-tRNA<sup>[Ser]Sec</sup> to the ribosome. eEFSec was discovered based on sequence similarity to its archaeal counterpart SelB. Much like SelB, eEFSec is a GTP binding protein with roughly equal affinity for GTP and GDP thus lacks the action of a guanine nucleotide exchange factor (GEF) for functionality [10, 11]. Unlike SelB, eEFSec does not bind to the SECIS element. Instead, experiments suggest eEFSec forms a transient complex with SECIS bound SBP2 and delivers Sec-tRNA<sup>[Ser]Sec</sup> to the ribosomal A-site during translation when the ribosome encounters a UGA codon in selenoprotein mRNA.

In contrast to eEFSec, the canonical translation elongation factor 1a (eEF1A) is the workhorse during protein elongation as it carries aminoacylated tRNAs to the ribosome to allow translation elongation. eEF1A contains 3 domains that are phylogenetically related to eEFSec. The function of the eEF1A domains have been characterized as such: Domain I is required for binding to the ribosome and guanosine-5'-triphosphate phosphatase (GTPase) activity, which drives binding of aminoacyl-tRNA in Domain II, and Domain III is specifically involved in binding the acceptor arm of tRNA as well as interacting with its GEF, eEF1B [48]. This characterization has provided some insight to the function of the similar domains in

eEFSec. However, it does not help explain the functional differences between the two elongation factors. The main functional difference between eEFSec and eEF1A begins with tRNA specificity, as eEFSec can only bind to Sec-tRNA<sup>[Ser]Sec</sup>, while eEF1A binds to the 20 canonically charged tRNAs. Analysis of the structure of archaeal SelB with X-ray crystallography revealed a chalice like structure, which has only been previously reported in IF2/eIF5B and not eEF1A [49]. While much is known about eEF1A, the precise roles for eEFSec domains remain largely unstudied and only speculation is currently possible based on similarity to eEF1A.

The most conspicuous difference between the two elongation factors is the additional unique domain on the C-terminal end of eEFSec, Domain IV, which has been implicated in all of the known functions for eEFSec: i) SBP2/SECIS binding, Sec-tRNA<sup>[Ser]Sec</sup> binding, and GTP hydrolysis [50]. The interaction between eEFSec and the SBP2/SECIS complex has only been observed in cells when tRNA<sup>[Ser]Sec</sup> was overexpressed [51], or when an electrophoretic mobility shift assay was used to capture the transient complex [39]. The fact that this interaction requires the presence of Domain IV may suggest that the SBP2/SECIS complex induces a stable conformational change in eEFSec that allows recognition of the UGA codon. This hypothesis remains to be tested.

GTP hydrolysis plays an important role in the proper function of canonical eEF1A and eEF2. However, the role GTP hydrolysis plays in eEFSec seems to be different. In eEF1A and eEF2, GTP hydrolysis is critical for the conformational changes required for stepwise progression through the elongation cycle. In eEF1A and eEF2, a GEF is used after hydrolysis to exchange GDP for GTP to allow for a conformational change to promote tRNA binding. The GTP bound form of SelB has a million-fold higher affinity for Sec-tRNA<sup>[Ser]Sec</sup> than the GDP bound or apo form. Upon binding GTP, SelB undergoes a conformational change and then a stabilization of the SelB/GTP/Sec-tRNA<sup>[Ser]Sec</sup> complex occurs [52]. It is thought that Sec-tRNA<sup>[Ser]Sec</sup> is delivered to the ribosomal A site by SelB in the presence of the SECIS element, and then SelB hydrolyzes GTP, which causes the rapid release of Sec-tRNA<sup>[Ser]Sec</sup> from eEFSec [53]. While bacterial SelB and eukaryotic eEFSec have many differences, it seems likely that the fundamental mechanism of Sec-tRNA<sup>[Ser]Sec</sup> accommodation in the ribosomal A-site may be conserved.

## 2.6 Other Factors

While the core essential factors involved in Sec incorporation have been shown to be sufficient for Sec incorporation [2], there are many other factors that are implicated in Sec incorporation either by direct experimental evidence or phylogenetic relationships. The most striking example of the latter is the SECIS binding protein 2 like protein (SECISBP2L). SECISBP2L was identified via BLAST searches based on its similarity to SBP2 in the C terminal domain where it shares a 46% amino acid identity [9]. Like SBP2, SECISBP2L has both a RNA binding domain

and Sec incorporation domain and can specifically bind to the AUGA core. SECISBP2L is not functionally active in Sec incorporation in vitro, and there currently is no direct evidence of any function [41]. The lack of a discernable function for SECISBP2L is a puzzle that is not illuminated by phylogenetic analysis of its origins, which seem to suggest that SBP2 and 2 L are orthologues that diverged after a gene duplication event during early evolution of vertebrates [41]. Additionally, vertebrate SECISBP2L seems to be more closely related to invertebrate SBP2 than vertebrate SBP2 based on the number of conserved regions found between them. Many deuterostomes do not have SBP2, but still retain SECISBP2L as the only SECIS binding protein, suggesting in some organisms that SECISBP2L is active in Sec incorporation [41]. Currently, it is believed that the divergence of SBP2 and SECISBP2L in vertebrates caused SECISBP2L to lose its ability to support Sec incorporation. While not functional for Sec incorporation in vitro, it is still possible SECISBP2L might serve some other undiscovered function in vivo. Despite the lack of direct evidence, however, it is striking that mice lacking *Secisbp2* retain substantial selenoprotein synthesis capacity [54], which is possibly supported by an as-yet, undetermined function for SECISBP2L.

Another non-essential SECIS binding protein identified, but not fully understood, is eukaryotic initiation factor 4a3 (eIF4A3). eIF4A3 is an RNA dependent ATPase, ATP dependent RNA helicase, and a DEAD-box protein family member that was found to regulate Sec incorporation [55]. While eIF4A3 is similar to the two other isoforms of eIF4A (I and II), it appears that is functionally distinct. In addition to its function in nonsense mediated mRNA decay [56], eIF4A3 binds to the *GPX1* SECIS element at both the internal and apical loop. Binding to SECIS by eIF4A3 prevents SBP2 binding and therefore inhibits Sec incorporation in vitro. It also appears to have a differential binding affinity, specifically to the *GPX1*, but not the *GPX4* SECIS element, giving it a role in regulating selenoprotein synthesis [57]. In McArdle 7777 rat hepatoma cells, eIF4A3 expression becomes upregulated in the absence of Se, and in turn reduced Gpx1 levels while Gpx4 levels were unaffected [55]. Thus, eIF4A3 has emerged as a potentially key factor in determining the hierarchy of selenoprotein expression when Se becomes limiting [reviewed in [58]].

Ribosomal protein L30 (RPL30) is another SECIS binding protein implicated in Sec incorporation [59]. It is a small (14.5 kDa) protein that is part of the large ribosomal subunit [60]. Like SBP2, RPL30 also contains an L7Ae RNA binding motif and competes with SBP2 binding to the SECIS element in vitro [59, 61]. Although RPL30 was shown to stimulate Sec incorporation in transfected cells, it is not yet known whether it is essential for Sec incorporation or whether it can bind to all SECIS elements. The SECIS binding activity of RPL30 is consistent with phylogenetic analysis, which concluded that the L7Ae motif of SBP2 arose from RPL30 [41]. RNase footprinting assays show the binding of SBP2 and RPL30 have some overlap on the SECIS element, but also have their own individual sites as well [61]. The current model of RPL30 activity is in promoting dissociation of the SBP2/SECIS complex by binding to SECIS allowing for canonical ribosomal elongation to continue.



## 2.7 Unique Sec Incorporation: Selenoprotein P

The plasma selenoprotein, selenoprotein P (SEPP1), is unique because it possesses multiple UGA codons in the mRNA. In humans and rats, there are 10 Sec residues, but it is highly variable between other eukaryotic species. Most of the Sec residues tend to be concentrated at the C terminal end of the protein, but the position of the first UGA codon is highly conserved. Another unique feature of the *SEPP1* mRNA is a long 800 nucleotide 3' UTR containing both types of SECIS elements, Form 1 and Form 2. In vitro translation experiments in rabbit reticulocyte lysate have demonstrated the efficiency of Sec incorporation into single UGA transcripts is relatively low at about 5–8 % [40]. With such a low efficiency, it would seem to be impossible to efficiently create proteins with multiple UGAs, but the 26 µg/ml of SEPP1 protein observed in plasma and the high SEPP1 levels found in tissue culture suggest that the efficiency is much higher in vivo (see [62] and Chap. 22). Indeed, recent work has indicated that the efficiency of Sec incorporation at UGA codons downstream of the first UGA is much more efficient [63]. Analysis of UGA redefinition in vivo using ribosomal profiling also supports a higher than 10 % UGA redefinition efficiency in hepatic selenoprotein biosynthesis (see Chap. 3 and [64]). Thus, two questions emerge when considering the requirements for SEPP1 synthesis: how is a protein with 10 Sec codons made efficiently, and how are the multiple Sec residues incorporated processively in order to generate full length SEPP1 protein (See also Chap. 3)?

It has been proposed that SEPP1 translation efficiency and processivity is regulated by *cis* acting factors in the 3' UTR of *SEPP1* involved with recoding of UGAs for Sec incorporation. Some of these *cis* elements were hypothesized in initial sequence analysis of *SEPP1* looking for conserved regions across species where two regions in the 3' UTR surrounding and including the two SECIS elements were found to be highly conserved [65]. It was hypothesized that the two SECIS elements were involved in processive Sec incorporation [66]; however, in vitro tests showed neither Form II SECIS nor most of the 3' UTR is required for *SEPP1* translation [63, 67]. Swapping the *SEPP1* 3' UTR with other selenoprotein 3' UTRs resulted in reduced efficiency, but processive production of full length SEPP1 protein in vitro was still observed, indicating clear separation of efficiency and processivity [63, 67].

## 2.8 Impact of In Vitro Translation Systems for Studying Sec Incorporation

Cell free in vitro translation systems have been key tools for investigators in determining the factors involved with Sec incorporation. The identification, validation and characterization of both SBP2 and eEFSec has in large measure taken place in two commercially available in vitro translation systems: rabbit reticulocyte and



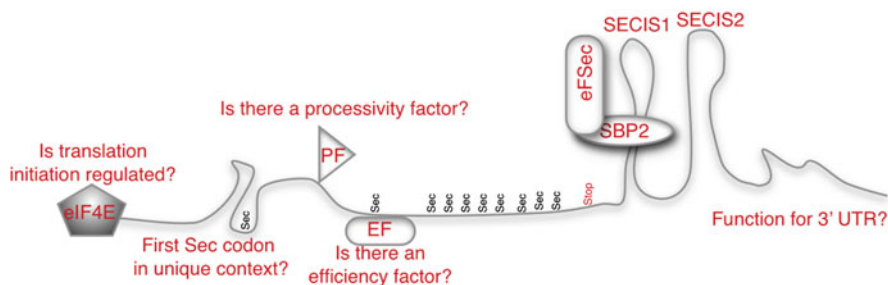
wheat germ lysates. Both of these systems have their uses and limitations, and have an established record of being reliable ways of solving some of the fundamental questions in translation. Prior to the discovery of SBP2, the rabbit reticulocyte lysate system was used to explore the ability of tRNA<sup>[Ser]Sec</sup> to suppress translation termination [68, 69]. The use of rabbit reticulocyte lysate expanded significantly when it was found that SBP2 is extremely limiting in the system, thus paving the way for the formal proof that SBP2 is necessary for Sec incorporation [9], and extensive characterization of its domain functions [25, 33, 39, 40, 70]. The physiological basis for the lack of SBP2 in this system is an interesting topic that has not been investigated. In the case of eEFSec, there is no evidence that the factor is limiting in rabbit reticulocyte lysate, and its biochemical characterization required the development of a new system.

The commercially available wheat germ lysate system was used in an attempt to reconstitute Sec incorporation with only the known factors, taking advantage of the fact that higher plants do not utilize Sec and do not have any of the Sec incorporation factors. Initially it was found that the addition of SBP2, eEFSec, Sec-tRNA<sup>[Ser]Sec</sup> and mammalian ribosomes was sufficient to promote robust Sec incorporation into a luciferase reporter construct harboring *SEPP1* 3' UTR [2]. Subsequent work with a different batch of wheat germ lysate demonstrated that mammalian ribosomes were not required [67], thus establishing that SBP2, eEFSec and Sec-tRNA<sup>[Ser]Sec</sup> are sufficient to promote Sec incorporation even with ribosomes that did not evolve to work with these factors.

The wheat germ lysate system has tremendous potential in terms of providing a test bed for all of the fundamental mechanistic questions surrounding both Sec incorporation and Sec-tRNA<sup>[Ser]Sec</sup> synthesis. For example, three different SECIS elements showed markedly different Sec incorporation efficiencies [2], consistent with those previously observed in rabbit reticulocyte lysate [33]. In addition, it was recently found that while full length SEPP1 protein can readily be made in rabbit reticulocyte lysate, only a single Sec incorporation event was observed in wheat germ lysate [67], thus providing strong evidence for the existence of an as yet unidentified factor required for processive Sec incorporation. As illustrated in Fig. 2.1, this wheat-based system is primed to answer several key questions regarding the mechanism of SEPP1 synthesis, and this is just one example of the extent to which our knowledge of the fundamental mechanism of Sec incorporation may be revealed.

## 2.9 Concluding Remarks

In the 25 years since the discovery of the SECIS element, great strides in understanding the mechanism and regulation of Sec incorporation in selenoproteins have been made, but questions still remain. The understanding of the essential Sec incorporation factors and their fundamental roles brings more insight to the complexity of selenoprotein regulation. Our greater understanding of these mechanisms has



**Fig. 2.1** An illustration of the many mechanistic questions that can be answered about the mechanism of SEPP1 synthesis using a wheat-germ lysate based *in vitro* translation system. The figure shows the SEPP1 mRNA containing ten Sec codons and two SECIS elements along with the known (SBP2, eEFSec) and proposed processivity (PF) and efficiency (EF) factors. The potential role of codon context, regulated translation initiation and a function for the conserved non-SECIS portions of the SEPP1 3' UTR are also depicted

provided more explanations to the sources of diseases previously unknown, and will continue to do so. Many questions remain in the basic mechanism of Sec incorporation involving eEFSec complex formation with SBP2/SECIS and Sec-tRNA<sup>[Ser]Sec</sup> delivery to the ribosome. We also do not know how Sec-tRNA<sup>[Ser]Sec</sup> is accommodated into the ribosome or the role and mechanism of GTP hydrolysis for eEFSec, nor the temporal sequence of events. It is also too early to know if all the factors required for the regulation of Sec incorporation *in vivo* have been discovered because the difference in efficiency between cell free extracts and tissue culture is not well understood. *In vitro* translation systems have helped immensely in shedding light on fundamental questions of Sec incorporation so that the molecular mechanism of Sec incorporation can be deciphered.

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## Selenium

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