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MECHANISM AND REGULATION OF SELENOPROTEIN SYNTHESIS

Donna M. Driscoll[1] and Paul R. Copeland[2]
[1]*Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195; email: driscod@ccf.org*
[2]*Department of Molecular Genetics, Microbiology and Immunology, UMDNJ–Robert Wood Johnson Medical School, Piscataway, New Jersey 08854; email: paul.copeland@umdnj.edu*

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■ **Abstract** Selenium is an essential trace element that is incorporated into proteins as selenocysteine (Sec), the twenty-first amino acid. Sec is encoded by a UGA codon in the selenoprotein mRNA. The decoding of UGA as Sec requires the reprogramming of translation because UGA is normally read as a stop codon. The translation of selenoprotein mRNAs requires *cis*-acting sequences in the mRNA and novel *trans*-acting factors dedicated to Sec incorporation. Selenoprotein synthesis in vivo is highly selenium-dependent, and there is a hierarchy of selenoprotein expression in mammals when selenium is limiting. This review describes emerging themes from studies on the mechanism, kinetics, and efficiency of Sec insertion in prokaryotes. Recent developments that provide mechanistic insight into how the eukaryotic ribosome distinguishes between UGA/Sec and UGA/stop codons are discussed. The efficiency and regulation of mammalian selenoprotein synthesis are considered in the context of current models for Sec insertion.

CONTENTS

INTRODUCTION

The element selenium (Se^{34}) was discovered in 1817 by Swedish chemist Jons Jacob Berzelius. The mineral had properties similar to tellurium (Te^{52}). Since tellurium was named after tellus, the Latin word for earth, Berzelius named the new element after Selene, the Greek goddess of the moon. Historically, selenium was regarded as a naturally occurring toxic agent, but this perspective has undergone a radical transformation in the past 50 years. Selenium is now known to be an essential trace element which exerts a number of important health benefits (48). The micronutrient has also attracted the attention of molecular biologists because it is incorporated into proteins as selenocysteine (Sec), the twenty-first amino acid (15, 49). Sec is encoded by a UGA codon in the selenoprotein mRNA. The decoding of UGA as Sec requires the reprogramming of translation because UGA is normally read as a stop codon. The UGA/Sec codon is the first addition to the universal genetic code since the code was discovered in the mid 1960s (91, 100).

The translation of selenoprotein mRNAs requires both *cis*-acting sequences in the transcript and novel *trans*-acting factors dedicated to Sec incorporation. The synthesis of selenoproteins in vivo is highly selenium-dependent, and there is a hierarchy of selenoprotein expression in mammals when selenium is limiting (1, 107). The mechanism of selenoprotein synthesis in prokaryotes was elucidated by the elegant work of Böck and colleagues [reviewed in (15, 16)], but our understanding of this pathway in eukaryotes lagged behind until recently. In this review, we provide a brief overview of the importance of selenium and selenoproteins in human health followed by a description of emerging themes from studies on the mechanism, kinetics, and efficiency of Sec insertion in prokaryotes. We also discuss recent developments that provide mechanistic insight into how the eukaryotic ribosome distinguishes between UGA/Sec and UGA/stop codons. Finally, the efficiency and regulation of this translational recoding event in mammals is considered in the context of current models for Sec insertion.

SELENIUM

In the late 1950s, it was established that selenium is an essential micronutrient (42). Based on metabolic labeling studies, most of the selenium in animals is associated with protein, with 80% incorporated as Sec in the selenoproteins (50). The dietary requirement for selenium is most likely due to its function in the selenoproteins whose synthesis is reduced when selenium is limiting. Because the role of selenium in human health and disease has been the subject of several recent reviews and book chapters (35, 42, 48, 49, 83, 84, 93), this topic is only briefly discussed here. Selenium deficiency has been implicated as a factor in Keshan disease, a cardiomyopathy that affects young women and children in certain regions of China that have selenium-poor soil. This disease can be prevented by dietary selenium supplementation, as can Kashin-Beck disease, a deforming osteoarthritis also found in China (24). In humans, moderate selenium insufficiency may contribute to the pathogenicity of viral infections (8), the progression of AIDS (7), male infertility (36), and impaired immunity (85). The trace element may also have a protective effect against inflammatory diseases such as arthritis, asthma, and atherosclerosis (93). Selenium has recently sparked a lot of interest as an anticancer nutrient. The results of animal experiments, epidemiological studies, and clinical intervention trials support the hypothesis that dietary selenium reduces the risk of certain types of cancers (23). Whether this protective effect is mediated by the selenoproteins or by low molecular weight selenium compounds is an important line of investigation for future research.

MAMMALIAN SELENOPROTEINS

Structure and Function of Selenocysteine

Proteins that contain a covalently bound Sec residue have been identified in all three lines of descent: prokaryotes, eukaryotes, and archaebacteria (49). Many of the characterized selenoproteins are enzymes that catalyze oxidation reduction reactions and contain Sec in their active site (104, 105). Structurally, Sec is identical to cysteine (Cys), except that it contains selenium instead of sulfur. Selenium is in the same family below sulfur in the periodic table, and the two elements share many similar chemical properties. Sec has a distinct functional advantage because the selenol group is more fully ionized than the thiol group of Cys at physiological pH (103). When Sec is replaced with Cys, the catalytic activity of a selenoenzyme is drastically reduced (2, 3, 11). Although selenoproteins have been identified in a wide variety of organisms, they are not ubiquitously expressed. There are no known selenoproteins in higher plants, or in yeast, an organism whose genome has been sequenced. In these organisms, the corresponding proteins contain Cys rather than Sec. This phenomenon leads to the question of whether these Cys-containing enzymes have evolved a compensatory mutation in the active site to make up for the loss in activity or whether higher activity is gained through dosage compensation.

Identification and Function of Mammalian Selenoproteins

The first Sec-containing protein in mammals was discovered in 1973 (95). Since that time, more than 20 eukaryotic selenoproteins have been identified (49). The knockout of the Sec-tRNA gene in mice resulted in early embryonic lethality (17), demonstrating that the function of one or more of these selenoproteins is essential for life. The selenoproteins with known functions play critical roles in a variety of biological processes, and several of them are involved in antioxidant defense. Two of the glutathione peroxidases (GPx) protect cells against peroxidative damage by reducing hydrogen peroxide and free fatty acid hydroperoxides (37). Another GPx family member, phospholipid hydroperoxide glutathione peroxidase (PHGPx), reduces phospholipid, cholesterol, and cholesteryl ester hydroperoxides, thereby protecting cells against membrane lipid peroxidation (37). PHGPx also plays a structural role in the mitochondrial capsule of mature spermatozoa where the protein becomes oxidatively cross-linked and inactive (113). This noncatalytic function of PHGPx may be responsible for the male infertility seen in selenium deficiency (36). In mammals, three distinct mammalian thioredoxin reductases function in cellular redox homeostasis by reducing thioredoxin and other substrates (55). Other oxido-reductases that contain Sec include the family of deiodinases, which are involved in thyroid hormone metabolism (102), and selenophosphate synthetase 2 (SPS2), which synthesizes the selenium donor for Sec biosynthesis. This enzyme is unique in that it is the only selenoprotein expressed in both prokaryotes and eukaryotes (46).

Several selenoproteins have no known enzymatic activity, including SelW, which is expressed in cardiac and skeletal muscle (120), and Sep15, which is implicated in preventing prostate cancer (43). Novel selenoprotein genes have been identified in the human genome using bioinformatic approaches, but the functions of the encoded proteins are largely unknown (66, 74). One of these orphan selenoproteins, SelR, was recently shown to be a methionine sulfoxide reductase (67). An antioxidant function has also been proposed for the plasma protein, selenoprotein P (SelP). The SelP mRNA encodes 10 to 17 UGA/Sec codons, depending on the species (53), and mass spectrometric analysis has verified that full-length rat SelP contains the 10 predicted Sec residues (78). Understanding how multiple UGA codons are decoded as Sec in SelP would provide important insight into the mechanism of selenoprotein synthesis.

TRANSLATIONAL DECODING OF UGA AS SELENOCYSTEINE

Unique Aspects of Sec Insertion Compared to Normal Protein Synthesis

Given that the nutritional requirement for selenium is most likely related to its function in the selenoproteins, it is important to understand how this class of proteins is synthesized. In prokaryotes, archaebacteria, and eukaryotes, Sec is encoded

by a UGA codon (49). The incorporation of Sec into protein requires a novel Sec-charged tRNA that contains the anticodon UCA (28, 73). Since UGA normally signals the termination of protein synthesis, how does the ribosome cope with a codon that encodes two different functions? Three unique aspects of selenoprotein mRNA translation distinguish it from normal protein synthesis. Sec is a remarkable amino acid in that it is universally synthesized on $tRNA^{Sec}$. Second, the decoding of UGA as Sec is dependent on *cis*-acting sequences in the selenoprotein mRNA. Last, due to unusual structural properties that prevent recognition by standard elongation factors, Sec-$tRNA^{Sec}$ is delivered to the ribosome by novel *trans*-acting proteins.

Sec is Synthesized on its tRNA

Genetic and biochemical studies have identified three gene products in *Escherichia coli* that are essential for the synthesis of Sec-$tRNA^{Sec}$ (15, 16). The $tRNA^{Sec}$ gene *selC* encodes a tRNA that is initially aminoacylated with serine by the normal serine synthetase (73). This serine residue provides the carbon backbone for Sec. The *selA* gene product, Sec synthase, converts the Ser-$tRNA^{Sec}$ to Sec-$tRNA^{Sec}$ in a two-step reaction that involves the loss of water and the addition of selenium (38, 40). The selenophosphate donor for this reaction is synthesized by the *selD* gene product, selenophosphate synthetase (68, 71).

The structure and synthesis of mammalian Sec-$tRNA^{Sec}$ has recently been reviewed in depth (22, 49). There are two major isoforms of mammalian Sec-$tRNA^{Sec}$ whose structures differ by a methyl group on U^{34}, the nucleotide in the first position of the anticodon (29, 61). Interestingly, the methylation of U^{34} appears to be regulated by selenium concentration, suggesting that the two isoforms may have different functions (29). In addition, Sec-$tRNA^{Sec}$ possesses an important modification at position 37 where an isopentenyladenosine residue is required for normal selenoprotein expression and relative distribution of the two isoforms at position 34 (86). The pathway for Sec synthesis is likely conserved in eukaryotes, although the mechanism is not fully characterized. It is presumed that the conversion of Ser-$tRNA^{Sec}$ to Sec-$tRNA^{Sec}$ is catalyzed by a Sec synthase, although this enzyme has not been identified in mammals. There are two selenophosphate synthetases, one of which (SPS2) is a selenoprotein (46, 77). The fact that a Sec-dependent enzyme synthesizes the selenium donor that is required for selenoprotein synthesis raises the intriguing possibility of feedback regulation.

SEC INCORPORATION IN PROKARYOTES

SelB, a Novel Elongation Factor Specific for Sec

$tRNA^{Sec}$ has several unusual structural properties, including an unusually long acceptor arm that is an antideterminant for binding to the standard elongation factor (EF)-Tu (4, 6, 96). During the decoding of UGA as Sec, the function of EF-Tu is carried out by SelB, a novel GTP-dependent elongation factor that is specific for

Sec incorporation (39, 41). The N-terminal region of SelB shares homology with EF-Tu, including the domains for tRNA binding and GTP binding. Unlike EF-Tu, SelB binds Sec-tRNA^{Sec} but not other aminoacylated tRNAs (39). A second mechanistic difference between the two elongation factors is that SelB has a dual function. In addition to binding Sec-tRNA^{Sec}, SelB binds to a specific sequence in the selenoprotein mRNA, which has been termed the bacterial Sec insertion sequence (bSECIS). The bSECIS is a stem-loop structure located immediately downstream of the UGA/Sec codon (58, 123). RNA footprinting studies showed that SelB binds to the apical loop region of the bSECIS element (5). The mRNA-binding activity of SelB is mediated by its unique C-terminal domain, which is not found in EF-Tu (65). Although the bSECIS-binding and tRNA-binding domains of SelB are physically distinct, some evidence suggests that the two domains may communicate (5).

Model for Sec Insertion: The Quaternary Complex

In normal protein synthesis, the aminoacylated tRNA is delivered to the ribosome by a ternary complex of EF-Tu, GTP, and the charged tRNA. After a specific codon:anticodon interaction occurs, GTP is hydrolyzed, EF-Tu undergoes a conformational change, and the charged tRNA is released to the ribosomal A site. EF-Tu/GDP then dissociates from the ribosome and the bound GDP is displaced by the GTP exchange factor. In contrast, the cotranslational incorporation of Sec requires the formation of a quaternary complex (see Figure 1). SelB forms a complex with GTP, the Sec-tRNA^{Sec}, and the bSECIS element in the selenoprotein mRNA (51). As the ribosome approaches during elongation, the lower part of the bSECIS stem-loop is partially unwound so that the quaternary complex is in position to deliver Sec-tRNA^{Sec} when the UGA codon reaches the ribosomal A site

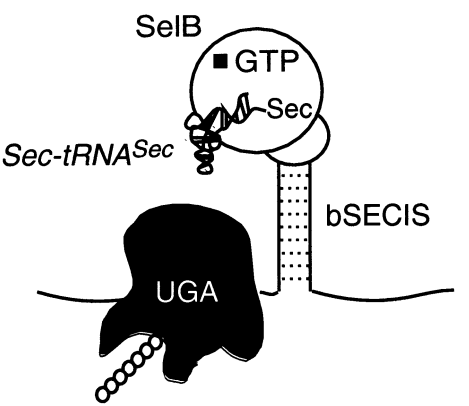

Figure 1 A model for Sec incorporation in bacteria. The schematic shows the formation of the quaternary complex with SelB, charged Sec-tRNA^{Sec}, GTP, and the bSECIS element in the selenoprotein mRNA.

(79). This tethered tRNA model is supported by in vitro and in vivo studies (5, 110). Importantly, the formation of the quaternary complex is more than just a docking event that increases the local concentration of factors. When SelB binds to the bSECIS element, the protein undergoes a conformational change, which renders it competent to associate with the ribosome (57, 63). Furthermore, the presence of ribosomes stimulates the GTPase activity of SelB, but only when this protein is bound to the bSECIS element (57). Thus, the quaternary complex is essential for a productive interaction with the ribosome, and consequently for the stimulation of GTP hydrolysis. After the Sec-tRNA^{Sec} is released to the ribosomal A site, the affinity of SelB for the bSECIS decreases, causing the SelB/GDP/bSECIS complex to dissociate (109). Because SelB has a higher affinity for GTP than for GDP, it does not need to bind the GTP exchange factor, and in fact the domain for this interaction, which is found in EF-Tu, is missing in SelB (39, 65).

Kinetics, Efficiency, and Regulation of Sec Insertion in *E. coli*

KINETICS AND EFFICIENCY The efficiency of Sec insertion in *E. coli* is quite low, ~4% compared to the incorporation of other amino acids (108). This inefficiency is not solely due to limiting components of the Sec incorporation machinery because increasing the concentration of the key players (i.e., SelB, tRNA^{Sec}, or Sec synthase) improved Sec incorporation only two- to three fold (79, 108, 110). Instead, binding of the SelB complex to the stem-loop structure in the selenoprotein mRNA induces a long translational pause when the UGA/Sec codon is in the ribosomal A site (108). Another important conclusion that emerged from this study is that the decoding of the UGA/Sec codon by the quaternary complex is an intrinsically slow process. What might limit SelB-dependent decoding? Suppmann et al. (108) proposed that the inefficiency of Sec incorporation may be related to one or more steps in the reaction, including the association of the SelB complex with the upstream ribosome, the hydrolysis of GTP, the release of Sec-tRNA^{Sec} from the complex, or the binding of Sec-tRNA^{Sec} to the ribosomal A site. A major challenge for future studies is to characterize these intermediate steps at the biochemical level.

CONCENTRATION OF FACTORS As mentioned above, selenoprotein synthesis is stimulated several-fold when the pool of Sec-tRNA^{Sec} is increased, suggesting that this molecule is a limiting factor. The levels of Sec-tRNA^{Sec} are also selenium-dependent. After charging with serine, Ser-tRNA^{Sec} is bound by the enzyme Sec synthase (41, 111). The serine is converted to Sec only when selenium is available to synthesize the selenophosphate donor. If selenium is limiting, there is no Sec-tRNA^{Sec} available for delivery to the ribosome, resulting in the termination of protein synthesis (15). The proper stoichiometry of the individual subunits is also essential for the formation of the quaternary complex, and an imbalance of factors can inhibit Sec insertion. Overproduction of SelB results in nonproductive interactions in which the protein is bound either to the Sec-tRNA^{Sec} or to the mRNA, but not to both (110). Likewise, an excess of selenoprotein mRNA will inhibit Sec

incorporation by swamping out essential *trans*-acting factors (72, 110). In both cases, a truncated product is produced because the ribosome terminates at the UGA/Sec codon, demonstrating that Sec insertion and termination are competitive events.

COMPETITION BETWEEN SEC INSERTION AND TERMINATION Since decoding by SelB is slow, what is the likelihood that termination will occur before Sec insertion? In prokaryotes, termination is catalyzed by two release factors (RFs) that exhibit stop codon specificity (13, 88). RF1 recognizes UAG and UAA, whereas RF2 recognizes UGA and UAA. Chemical cross-linking studies showed that this recognition is achieved by direct physical contact between the RF and the appropriate stop codon (20, 69). The RF can also be cross-linked to the fourth base of the stop codon, which explains why this nucleotide influences termination efficiency (92). Because Sec insertion and termination are in a reciprocal relationship, the decoding of UGA as Sec should be more efficient when the fourth nucleotide of the stop codon is in an unfavorable context for termination, i.e., UGAA or UGAC (92). The current model for termination is based on the hypothesis that RFs are structurally and functionally similar to tRNA molecules (59, 90). In the tRNA mimicry model, the RF binds to the ribosomal A site in response to a stop codon and catalyzes the release of the nascent polypeptide chain (13, 88). Since RF2 and Sec-tRNA^{Sec} compete for the same binding site on the ribosome, changing the relative cellular concentrations of these two factors modulates whether UGA is decoded as Sec or stop (79). Interestingly, the incorporation of Sec is more efficient at lower growth rates, which presumably is an advantage because some bacterial selenoproteins are only expressed under anaerobic conditions when the growth rate is slower (79). Although the molecular basis for this phenomenon is not known, one can imagine that certain conditions regulate the levels or subcellular localization of the *trans*-acting factors involved in Sec insertion, and consequently the rate of quaternary complex assembly.

MECHANISM OF SEC INSERTION IN EUKARYOTES

Although there is almost no overlap between the selenoproteins expressed in prokaryotes and eukaryotes, the mechanism for Sec incorporation is partially conserved between the two kingdoms. Sec insertion in eukaryotes also requires a stem-loop structure in the selenoprotein mRNA as well as a Sec-specific elongation factor. A series of remarkable discoveries over the past decade revealed that the eukaryotic selenoprotein biosynthetic pathway has evolved several unique features that provide a higher level of complexity and greater potential for regulation (see Figure 2).

Cis-Acting Sequences: The SECIS Element

The first unexpected finding was that the eukaryotic SECIS element is located in the 3′ untranslated region (3′ UTR) of the mRNA (9). The distance between

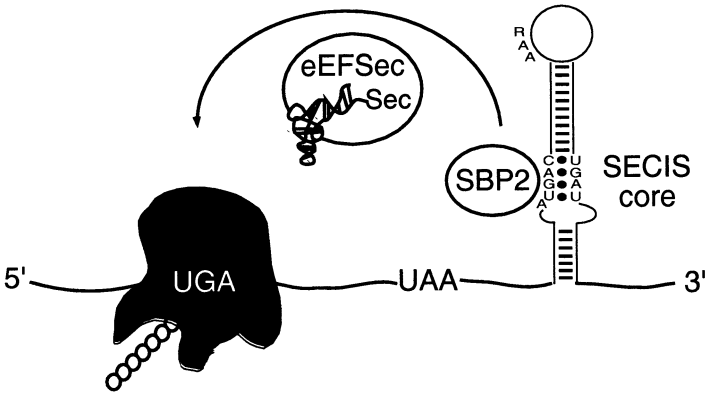

Figure 2 A model for Sec incorporation in eukaryotes. The schematic shows a representative SECIS element that contains a quartet of non–Watson-Crick base pairs in the SECIS core. The known *trans*-acting factors involved in Sec insertion (Sec-tRNA^{Sec}, eEFSec, and SBP2) are indicated.

the UGA/Sec codon and the SECIS element in eukaryotic selenoprotein mRNAs can be quite far, in some cases over 4000 nucleotides (21). In archaebacteria, the SECIS element is also located in the 3′ UTR, which suggests that the archaeal and eukaryotic mechanisms for Sec insertion are similar (121). There are several advantages to having the SECIS in the 3′ UTR rather than in the coding region. The evolution of the amino acid sequence is not constrained by the need to maintain a stem-loop structure in the mRNA. Second, the eukaryotic SECIS stem-loop does not need to unfold and refold during translation. Finally, the SECIS element may be able to regulate the translation reaction with more flexibility from the 3′ UTR, as is the case for the 3′ poly (A)/poly(A) binding protein complex (97). This flexibility may be manifested in the fine regulation of the efficiency and processivity of Sec incorporation, as discussed below.

Eukaryotic SECIS elements are not highly conserved at the nucleotide level but they all form a similar stem-loop structure, which is composed of two helices separated by an internal loop (80). The SECIS core, which lies at the junction of helix 2 and the internal loop, contains a quartet of non–Watson-Crick base pairs (see Figure 2). The center of this quartet forms a G.A./A.G. base pair tandem, which is conserved in all eukaryotic selenoprotein mRNAs (114, 115). SECIS elements can be divided into two classes, depending on the position of a conserved AAR motif, which is found either in the apical loop or in an internal loop (33, 44). Mutagenesis of the purines in the SECIS core or the AAR motif abolished selenoprotein synthesis, demonstrating the functional importance of these nucleotides (80). Although it was believed that the AAR motif was invariant, a recent study identified a novel selenoprotein, SelM, that contains a noncanonical SECIS element in which the A residues are replaced with cytidines (64). Thus, the SECIS element is a highly conserved structure that likely provides the platform for the protein factors required for Sec incorporation. Interestingly, the SECIS element is strikingly similar

to the recently classified kink-turn motif, which is a relatively common element found in the large subunit rRNA (62). One or more of these elements may therefore be directly interacting with SBP2 and thus anchoring it to the ribosome (see below).

Trans-Acting Proteins

SECIS-BINDING PROTEIN 2 By analogy with the prokaryotic system, a number of labs searched for RNA-binding proteins using the SECIS element as bait. Several candidate SECIS-binding proteins were identified in the past few years (31, 56, 75, 98, 99), but in most cases, the specificity of the interaction and the functional relevance to Sec insertion were not established. Compelling evidence has been presented for one protein, SECIS-binding protein 2 (SBP2). SBP2 is a novel protein that contains an RNA-binding motif, which is found in eukaryotic release factor 1 (eRF1) and ribosomal proteins (26). Mutagenesis studies demonstrated that this domain is required for the SECIS-binding activity of SBP2 (27). Based on RNA-footprinting and chemical interference studies, the SBP2 binding site is centered on both sides of the SECIS core, with additional protection along the 5′ part of the lower helix (34). Immunodepletion experiments established that SBP2 is essential for Sec incorporation in vitro (26). It is likely that SBP2 is required for selenoprotein synthesis in vivo, but this hypothesis remains to be tested. SBP2 is widely expressed, with the highest levels found in the testis, presumably because this tissue needs to synthesize large amounts of PHGPx, a structural selenoprotein in mature spermatozoa (26). Interestingly, SBP2 has been demonstrated to stably interact with ribosomes in vitro and in cells, suggesting that SBP2 may directly modify the coding potential of the ribosomes to which it is bound (27). However, there is no evidence that the ribosome-binding activity of SBP2 is required for Sec incorporation.

SBP2 possesses a unique N-terminal domain and a C-terminal domain that is sufficient for all of the known functions of SBP2: SECIS binding, ribosome binding, and in vitro Sec incorporation (26, 27). As mentioned above, the "core" of the RNA-binding domain is similar to that found in some ribosomal proteins (e.g., L30) and the translation release factor eRF1. Mutations in the conserved glycine residue (SBP2 G669) eliminate SECIS binding and Sec incorporation (27). This mutation does not, however, affect ribosome binding, which suggests that the SECIS-binding and ribosome-binding domains are not identical. This is surprising considering that the ribosome interaction appears to be occurring through the large subunit rRNA. Further analysis of the amino acid requirements for each process is required before the precise definition of domain function can be assigned. Although SBP2 is clearly involved in Sec insertion, it quickly became evident that this protein does not function in Sec-tRNA^{Sec} delivery. SBP2 does not share any homology with elongation factors, and it does not bind the Sec-tRNA^{Sec} in vitro. The first key to solving this puzzle was provided by the discovery of the archaeal homolog of SelB in the *Methanococcus jannischii* genome (94).

eEFSEC, A SEC-SPECIFIC ELONGATION FACTOR By screening databases with the archaeal SelB sequence, two laboratories independently identified a mammalian Sec-specific elongation factor, termed mSelB or eEFsec (32, 112). The latter name is used in this review. Homologs of eEFsec were also found in the genomes of *Drosophila melanogaster* and *Caenorhabditis elegans*. Like SelB, eEFsec specifically binds the charged Sec-tRNA^{Sec} in vitro and in vivo (32, 112). eEFsec is also missing the domain for interaction with the GTP-exchange factor, which is consistent with the fact that eEFsec has a higher affinity for GTP than for GDP. Coimmunoprecipitation experiments showed that eEFsec is in a complex with SBP2 in transfected cells (112). Complex formation was reduced by RNase treatment, which suggests the involvement of an RNA component. The most intriguing discovery was that the archaeal SelB and eEFsec do not specifically bind to the SECIS element on their own (32, 112). Thus, in eukaryotes, the SECIS-binding and tRNA-binding functions of SelB are divided between two proteins, eEFsec and SBP2. In vitro binding studies suggest that eEFsec may bind to the SECIS through SBP2 or other unidentified factors (32). Interestingly, no archaeal homolog of SBP2 has been identified in the *Methanococcus jannischii* genome.

Models of Sec Insertion in Eukaryotes

By analogy with the prokaryotic system, it has been proposed that eukaryotic selenoprotein biosynthesis involves a complex of the SECIS element, SBP2, eEFSec, and Sec-tRNA^{Sec} (12). This model requires a looping out of the mRNA so that the loaded SECIS element in the 3′ UTR is positioned at the upstream UGA/Sec codon. Indirect evidence suggests that SBP2 remains stably associated with the SECIS element through multiple rounds of translation (76). In this situation, after delivering the Sec-tRNA^{Sec} to the ribosome, the empty eEFsec could then bind another charged tRNA^{Sec} for the next round of translation. Although the loopout model is reasonable and supported by the fact that the distance between the UGA and the SECIS elements does not appear to be a critical determinant for Sec insertion, it does not explain how the loaded SECIS locates the upstream ribosome. There is also no evidence that SBP2 binds to the 5′ UTR or the coding region of the selenoprotein mRNA. The issue is further complicated by the fact that the SelP mRNA encodes between 10 and 17 Sec residues in different species. If delivery of the loaded SECIS is stochastic, how are multiple UGA codons in a single transcript efficiently decoded as Sec?

IS SEC INSERTION PROCESSIVE? One possibility is that Sec insertion may be processive, where processivity is defined as the ability of the ribosome to become committed to decoding UGA as Sec instead of stop. We previously proposed that a subclass of ribosomes might be enriched in the translation factors required for Sec insertion. SBP2 has a ribosome-binding activity that is mediated by the 28S rRNA (27). Since SBP2 can self-associate, the ribosome-associated SBP2 may dimerize with the SECIS-bound SBP2 and recruit the selenoprotein mRNA to the Sec-competent ribosome. A second scenario for processivity involves a Sec-specific

initiation event. In light of the circular model of translation in which the 5′ and 3′ ends of an mRNA are in close proximity (119), the loaded SECIS element may recruit the necessary *trans*-acting factors to the ribosome as it loads on the mRNA. Alternatively, the ribosome may be reprogrammed during the first decoding event. The SBP2/SECIS complex may recruit the necessary *trans*-acting factors, which remain associated with the ribosome during elongation. SBP2 may even induce a change in the conformation of the ribosomal A site to increase its affinity for Sec-tRNA^{Sec}, a structurally unusual molecule. To date, the only study to systematically address processivity concluded that Sec insertion is nonprocessive (89). However, these experiments were not done with the SelP 3′ UTR, which contains two SECIS elements and naturally allows multiple Sec decoding events. In addition, this study used a dicistronic dual reporter construct with multiple artificial codons, and it is likely that the coding region, especially the Sec codon context, is important for processive Sec incorporation. The recent development of an in vitro system for translating selenoprotein mRNAs (26) should allow this hypothesis to be directly tested in the near future.

CRITICAL UNANSWERED QUESTIONS ABOUT MECHANISM In spite of the tremendous progress that has been made, a number of vital questions about the mechanism of Sec insertion have not yet been answered (see Figure 3). Does SBP2 actively

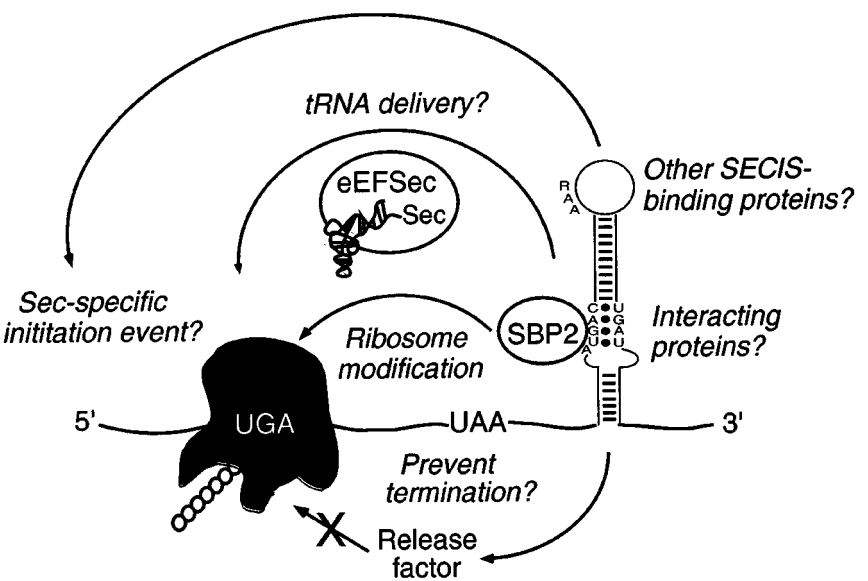

Figure 3 Critical unanswered questions about the eukaryotic mechanism of Sec insertion. The schematic shows steps in the eukaryotic pathway that need further investigation.

prevent termination at the UGA/Sec codon by sequestering RF or by sterically hindering the access of RF to the ribosomal A site? This is an attractive hypothesis because the RNA-binding domain of SBP2 shares homology with eRF1, which functions in termination (26, 27). Are other *trans*-acting factors required for Sec insertion? SBP2 exists in a high-molecular-weight complex in mammalian cells (25), but this complex has not been characterized in terms of composition or function. Additional proteins may interact with the SECIS element or with SBP2. What is the function of the AAR motif in the SECIS which is required for Sec insertion but not for SBP2 binding? These nucleotides could be involved in docking the loaded SECIS to the upstream UGA/Sec codon or in recruiting additional factors.

EFFICIENCY OF EUKARYOTIC SEC INSERTION

Because of inherent competition with the translation termination reaction, one of the central mechanistic questions regarding Sec incorporation is that of efficiency. The success of Sec incorporation is likely determined both by positive effects (those that specifically promote Sec incorporation) and negative effects (those that specifically promote termination). We first consider whether the efficiency of eukaryotic Sec insertion is influenced by parameters that modulate termination, and whether mammalian selenoprotein synthesis is efficient in vivo. The role of limiting factors in regulating efficiency is discussed in the next section.

Effect of Codon Context on Sec Insertion Efficiency

Early studies on the role of termination in regulating Sec incorporation showed that the base following the UGA codon (the fourth base) is a critical determinant of termination efficiency, which inversely correlates with the efficiency of Sec incorporation. Termination efficiency corresponds to the hierarchy A > G > C > U, where UGAA terminates most efficiently and UGAU the least. A concomitant analysis of deiodinase 1 translation in transfected cells showed that Sec incorporation has a preference for UGAC and UGAU, confirming the hypothesis that lower termination efficiency results in higher Sec insertion (82). These results were confirmed and expanded in more recent studies, which also addressed the role of upstream sequences (45, 89). In one case it was found that placement of an Asp and an Arg at the positions just upstream of the Sec codon limited Sec incorporation by over a hundredfold (89). This was an expected result as these codons have previously been shown to result in strong termination (14). Overall, it is clear that codon context plays a determinitive role in Sec incorporation. The full extent of the effect remains unknown because an exhaustive study of all possible contexts surrounding the Sec codon has not been done.

Since the fourth base identity has consistently been shown to be important, one might expect a codon bias to exist in nature to accommodate Sec incorporation, but our survey of 27 human Sec codons (10 from SelP and 17 from other selenoproteins) revealed that 56% possess a purine, usually G, at the fourth position (P.R.

Copeland, unpublished observation). SelP provides a good opportunity to evaluate the importance of fourth base contribution because the rat and human SelP mRNAs contain 10 Sec codons. In addition, there are four naturally occurring isoforms found in rat serum which correspond to termination at the second, third, seventh, and UAA^{ter} codons, indicating that termination and Sec incorporation occur with varying efficiency within the same mRNA (78). Interestingly, the Sec codons at these positions are followed by either a U or C. The eighth and ninth codons, which are followed by an A and G, respectively, do not yield stable truncated products, which suggests that they are not used as termination codons in normal human SelP synthesis. Clearly, the efficiency of termination as determined by the fourth base is not sufficient to explain the success or failure of Sec incorporation. This issue is further complicated by a recent study which showed a threefold *increase* in selenoprotein expression when the translation termination release factor eRF1 was overexpressed in mammalian cells (45). Although it was suggested that this surprising result might be explained by the sequestration of free eRF3 by the excess eRF1, the concomitant overexpression of eRF3 in this study did not reverse the effect.

Is Sec Incorporation Efficient In Vivo?

Much of the data regarding efficiency have suggested that Sec incorporation is often precluded by termination. Paradoxically, there is ample evidence that large quantities of selenoproteins are made in vivo, particularly in the testis. So the question arises: Is Sec incorporation inherently inefficient, with some compensatory mechanism in certain tissues, or is Sec incorporation efficient in vivo, but sensitive to manipulation in vitro? Clearly the latter is more likely to be true, but a true measure of efficiency is not easily obtained. The most rigorous definition of translational efficiency is, of course, the amount of protein produced per unit of mRNA. Because of the dramatically dynamic nature of mRNA and protein turnover, this is a difficult value to obtain, and the work published to date does not directly address the translational efficiency of selenoprotein synthesis. One recent report suggests that the efficiency of PHGPx translation is two times higher than that of GPx (117), but since differences in protein stability were not studied, a true assessment of efficiency was not made. The case of Sec incorporation is unique in that one can specifically address efficiency by comparing the translational yield of a wild-type selenoprotein with one in which Cys is substituted for Sec. In this case, differential protein stability is not likely to be a factor, and one can easily control for differential RNA stability by expressing data as a function of steady-state mRNA levels. However, it has been reported that excess selenoprotein mRNA can be a potent inhibitor of Sec incorporation (26). This is not surprising considering that the components of the Sec incorporation complex will in effect be "diluted" by excess mRNA. Therefore, any attempt to compare the translation of a selenoprotein mRNA with that of a Cys mutant must be done in the context of limiting mRNA, but the studies to date were performed in transiently transfected

cells where the selenoprotein biosynthetic machinery is likely to be swamped out. Although the question of efficiency remains unanswered, it is likely to be indirectly addressed in the context of the regulation of selenoprotein expression, both by virtue of selenium availability and by selective recruitment of limiting factors.

REGULATION OF MAMMALIAN SELENOPROTEIN SYNTHESIS

Limiting Factors as a Key Point of Regulation

In any multifactorial system, a key point of regulation is likely to be the provision of a limited supply of one or more essential factors. This certainly appears to be the case for Sec incorporation, but detailed information on which factors are limiting under which conditions is still lacking. Prior to the discovery of SBP2 and eEFsec, the only manipulable *trans*-acting factor was the Sec-tRNA^{Sec}. Several studies have shown that transfection of the tRNA^{Sec} gene increases Sec incorporation significantly in some cell culture experiments (10, 76) but not in others (87). In animals, however, it is clear that Sec-tRNA^{Sec} is not limiting. Mice that are heterozygous for the tRNA^{Sec} gene do not have reduced levels of GPx-1, despite having 50–80% less Sec-tRNA^{Sec} expressed (17), and overexpression of the wild-type Sec-tRNA^{Sec} does not affect selenoprotein expression (86). In addition to adequate selenium concentrations, the production of charged Sec-tRNA^{Sec} also requires the activity of the enzyme SPS2, and overexpression of SPS2 in mammalian cells does result in increased selenoprotein expression (19, 77).

In our initial work with SBP2, it was clear that the protein was limiting in reticulocyte lysates translation extracts (26) since the addition of recombinant SBP2 enhanced PHGPx translation ~20-fold. However, the stable transfection of SBP2 into the rat hepatoma cell line McArdle 7777 resulted in only a slight increase (less than twofold) in endogenous PHGPx protein levels (P.R. Copeland & D.M. Driscoll, unpublished observation). Whether SBP2 and eEFsec are limiting factors in transiently transfected cells has been investigated, but the published data are conflicting. For example, overexpression of SBP2 has been shown to enhance (76) or inhibit (112) Sec insertion in different studies from the same group. Likewise, it was concluded that eEFsec is limiting (32) or not limiting (112). Based on the available data, we conclude that both eEFsec and SBP2 are potentially limiting, but that the effects of adding SBP2 appear to result in more dramatic increases in Sec incorporation. It is likely that other, as of yet undiscovered, factors may also be limiting, which would give varying results depending on the cell type and the SECIS element employed. What is clear, however, is that an imbalance of factors can inhibit Sec insertion in mammalian cells, as it does in *E. coli* (76).

Regulation by Selenium

SELENIUM STATUS AFFECTS SELENOPROTEIN SYNTHESIS Although selenium is generally not limiting for most animals, the situation does arise under certain

circumstances. Of particular interest in the laboratory is that the supplementation of cell cultures with selenium results in significant increases in selenoprotein synthesis (34, 52). Selenium supplementation raises the level of charged Sec-tRNA^{Sec}, which allows for more efficient Sec incorporation (60); it also increases the loading of ribosomes on PHGPx and GPx mRNAs, indicating an increase in translation efficiency (34). Interestingly, the levels and activity of SBP2 are not selenium-regulated, in spite of this protein being a potentially limiting factor (P.R. Copeland & D.M. Driscoll, unpublished observation).

HIERARCHY OF SELENOPROTEIN SYNTHESIS The effect of selenium status in animals has also been studied by feeding the animals selenium-restricted diets. Under conditions of low selenium, GPx and SelP levels were drastically reduced. This loss of activity was the result of a concomitant loss of protein, which correlated with a loss of mRNA. Interestingly, it was noted that the loss of GPx activity was more severe than that for SelP (54), which suggests that individual selenoproteins may respond in unique fashions during selenium deprivation. This phenomenon has been described as the "hierarchy" of selenoprotein expression, particularly with regard to the glutathione peroxidase family. It has been noted that there are major differences in the loss of expression of different family members during selenium depletion as well as major differences in the rates at which these proteins are resynthesized during selenium repletion [reviewed in (18)]. Additional studies have confirmed that mRNA decay is a major determinant of selenoprotein expression during selenium depletion [reviewed in (1, 18)].

REGULATION OF SELENOPROTEIN mRNA STABILITY BY SELENIUM STATUS Most mRNAs containing premature stop codons are substrates for rapid decay by means of a process termed nonsense mediated decay (NMD). Since most, if not all, selenoprotein mRNAs fall into the category of potentially good substrates for NMD (i.e., they fit the rules for being subject to NMD) (106), it is perhaps not surprising that mRNA decay is a factor in the regulation of selenoprotein expression. However, some selenoprotein mRNAs are resistant to NMD even under conditions of limited selenium (70). It is tempting to suppose that SBP2 might be having a direct affect on mRNA stability by behaving in a similar fashion to the iron responsive protein (IRP-1) which binds to and stabilizes the transferrin mRNA (30). In this event, SECIS elements with the highest affinity for SBP2 would be more stable than those with lower affinity. Currently, there are no data to support this model. Since the SECIS element alone is not sufficient to alter mRNA stability (118), it is more likely that variable mRNA stability is a direct result of the efficiency of translation termination which is regulated both by the codon context and by the efficiency of Sec incorporation. This hypothesis is clearly supported by analyses of NMD in yeast where components of the termination complex are known to interact with factors required for NMD (116). Based on this model, one would predict that when translation results in Sec incorporation, selenoprotein mRNAs are stable and when translation results in termination, they are unstable. However, even this model is

too simple, as it has been shown that GI-GPx mRNA is actually increased during selenium deficiency even though the protein decreases to undetectable levels (122). To account for this fact, one must argue that stable selenoprotein mRNAs retain ribosomes queued upstream of the Sec codon in a configuration that is unable to allow termination. While polysome analysis has been performed on selenoprotein mRNAs (34, 81), these studies were not designed to illustrate the differences in polysome loading between an endogenous efficiently terminating message versus one that incorporates Sec. This experiment would require the stable transfection of cDNAs that contain either a Sec codon or a non-UGA stop codon. The difference in polysome loading should indicate the amount of ribosome pausing that may occur on different selenoprotein mRNAs, and a correlation may be established between stalled ribosomes (lack of termination) and increased mRNA stability.

DOES SBP2 REGULATE THE HIERARCHY OF SELENOPROTEIN EXPRESSION? As a limiting factor, SBP2 is likely to play a pivotal role in the regulation of selenoproteins. Under conditions of limiting selenium, both SBP2 and charged Sec-tRNA^{Sec} would be limiting. The establishment of the hierarchy of selenoprotein expression would be further complicated by the differential affinity or preference for eEFsec binding to the ribosomal A site. Therefore, we only consider the situation of a selenium replete organism—a realistic condition for the vast majority of human beings, if not all mammals. It would seem likely that, under conditions of limiting SBP2, differential SECIS affinity would be the primary determinant of selective selenoprotein expression. However, it has been reported that the difference in SBP2 binding affinity among six different SECIS elements spans a factor of only two-fold. It is, of course, possible that the affinities observed in vitro may not reflect those regulating binding in vivo. Alternatively, SBP2 may participate in the hierarchy of selenoprotein expression through a function other than its SECIS-binding activity, such as its potential role in regulating the efficiency of termination. Finally, there is also room for the existence of a separate factor, which may influence SBP2 affinity in vivo, or which also may be limiting and acting "downstream" of SBP2. While no such factor has been identified, this possibility cannot be excluded until an efficient cell-free system is derived from non–Sec-containing organisms such as *Saccharomyces cerevisiae*, in which efficient selenoprotein synthesis can be reconstituted with recombinant reagents.

CONCLUSIONS AND PERSPECTIVES

The study of selenoprotein synthesis allows us to consider several divergent areas: the import and management of a highly reactive trace element, the intricacies of translation elongation and termination, and the evolution of an essential biological system. This latter point is of particular interest in light of the fact that whole classes of organisms appear to have lost the ability, or never acquired the ability, to synthesize selenoproteins. Eventually, studies of molecular evolution should resolve this issue so that we may know whether mammalian systems are in the

process of getting rid of the selenium requirement or whether they are expanding their need for selenoproteins. With regard to mechanistic issues, we look forward to a complete understanding of Sec incorporation so that the system may be easily manipulated toward the fine-tuning of beneficial or harmful selenoprotein expression. This may become particularly important as a therapeutic approach against diseases that are the result of accumulated cellular injury. Finally, it should be noted that Sec is not the only nonstandard amino acid that is genetically encoded. Two recent studies provided convincing evidence that pyrrolysine is inserted in response to a UAG stop codon in eubacteria and archaebacteria (47, 101). The identification of pyrrolysine as the twenty-second amino acid is an exciting discovery but how the UAG stop codon is redefined is not understood. Studies on the mechanism of selenoprotein biosynthesis may provide insight into the decoding of UAG as pyrrolysine as well as other translational recoding events.

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CONTENTS