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Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons

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We investigated the requirements for selenocysteine insertion at single or multiple UGA codons in eukaryotic selenoproteins. Two functional SECIS elements were identified in the 3' untranslated region of the rat selenoprotein P mRNA, with predicted stem-loops and critical nucleotides similar to those in the SECIS elements in the type I iodothyronine 5' deiodinase (5'DI) and glutathione peroxidase selenoprotein mRNAs. Site-directed mutational analyses of three SECIS elements confirmed that conserved nucleotides in the loop and in unpaired regions of the stem are critical for activity. This indicates that multiple contact sites are required for SECIS function. Stop codon function at any of five out-of-context UGA codons in the 5'DI mRNA was suppressed by SECIS elements from the 5'DI or selenoprotein P genes linked downstream. Thus, the presence of SECIS elements in eukaryotic selenoprotein mRNAs permits complete flexibility in UGA codon position.

Key words: SECIS element/selenocysteine/UGA

Introduction

Translation of a UGA codon to give a selenocysteine residue requires specific components of the translational machinery, tRNAs with anticodons that base-pair with UGA and enzymes that produce tRNAs charged with selenocysteine. This process also requires a mechanism for recognizing the appropriate UGA codons and distinguishing them from UGA stop codons. Use of UGA as a selenocysteine codon occurs in both prokaryotes and eukaryotes, but the mechanism of recognition differs between these two kingdoms. Studies by Bock and coworkers showed that in the bacterial selenoenzyme, formate dehydrogenase (fDH), a specific stem-loop immediately 3' to the UGA codon is required for translation of this UGA as selenocysteine. Mutagenesis studies showed that the position of the stem-loop, the sequence of the loop and the ability of the stem to base-pair contribute to the interaction of this structure with the selenocysteine specific translation factor, SEL B (Bock *et al.*, 1991; Heider *et al.*, 1992). The stem-loop is in the coding region of the mRNA, and sequences in the 3' untranslated region (3' UTR) are not required for selenocysteine insertion. This is in contrast to the situation in eukaryotic selenoproteins. Our previous studies identified regions in the 3' UTRs of the cDNAs for the eukaryotic selenoproteins, Type I iodothyronine deiodinase (5'DI) and glutathione peroxidase (GPX), which are required for recognition of UGA as a selenocysteine codon (Berry *et al.*, 1991a). These regions, which we have termed SECIS (selenocysteine insertion sequence) elements, are predicted to form stem-loops with high negative free energies. We previously identified conserved features in these elements, including specific nucleotides in the loops and in unpaired regions of the stems. Deletions of 8-9 bp in the 5'DI and GPX loops abolished their ability to confer selenocysteine codon recognition (Berry *et al.*, 1991a). Recently, sequences of the cDNAs for rat and human selenoprotein P (Sel P) were reported (Hill *et al.*, 1991, 1993). These sequences are unique in that they each contain 10 in-frame TGA codons, and amino acid analysis of rat Sel P indicates a composition of ~8 mol Se per mol protein. The function of this protein, which is synthesized in the liver and secreted into the plasma, is unknown. Computer analysis of the long 3' UTR region in the rat Sel P cDNA sequence identifies numerous potential stem-loops, but only two of these have features in common with the 5'DI and GPX SECIS elements (Hill *et al.*, 1993, and this work). To date, no functional analysis of the Sel P 3' UTR has been reported.

The cDNAs for the 5'DI and GPX proteins each contain a single in-frame UGA codon, and one can postulate mechanisms whereby the stem-loop structures in these mRNAs interact with components of the translational machinery (selenocysteyl-tRNA and elongation factor) and possibly with RNA structures or the nascent protein in the environment of the UGA codon to specifically direct insertion of selenocysteine at this codon. However, it is difficult to envision how such a mechanism might work for the multiple UGA codons of Sel P. The rat 5'DI coding sequence terminates with a UAG; the human 5'DI, rat GPX and rat and human Sel P sequences all end in UAA. Thus, termination at the correct position is conferred by use of stop codons other than UGA. To gain insight into the mechanism of multiple UGA codon recognition, we examined the putative Sel P SECIS elements for their ability to confer translation of UGA as selenocysteine in the rat 5'DI coding region. We combined these with further studies which delineate the role of specific conserved nucleotides and secondary structural features in SECIS function. We also investigated the requirement for specific UGA codon contexts. Finally, the effects of SECIS element position on function were examined.

Results

Selenoprotein P contains two functional SECIS elements

Predicted secondary structures of the two putative Sel P SECIS elements, along with those of the rat 5'DI and GPX, are shown in Figure 1. Comparison of the secondary structures and alignment of the sequences (Figure 2)

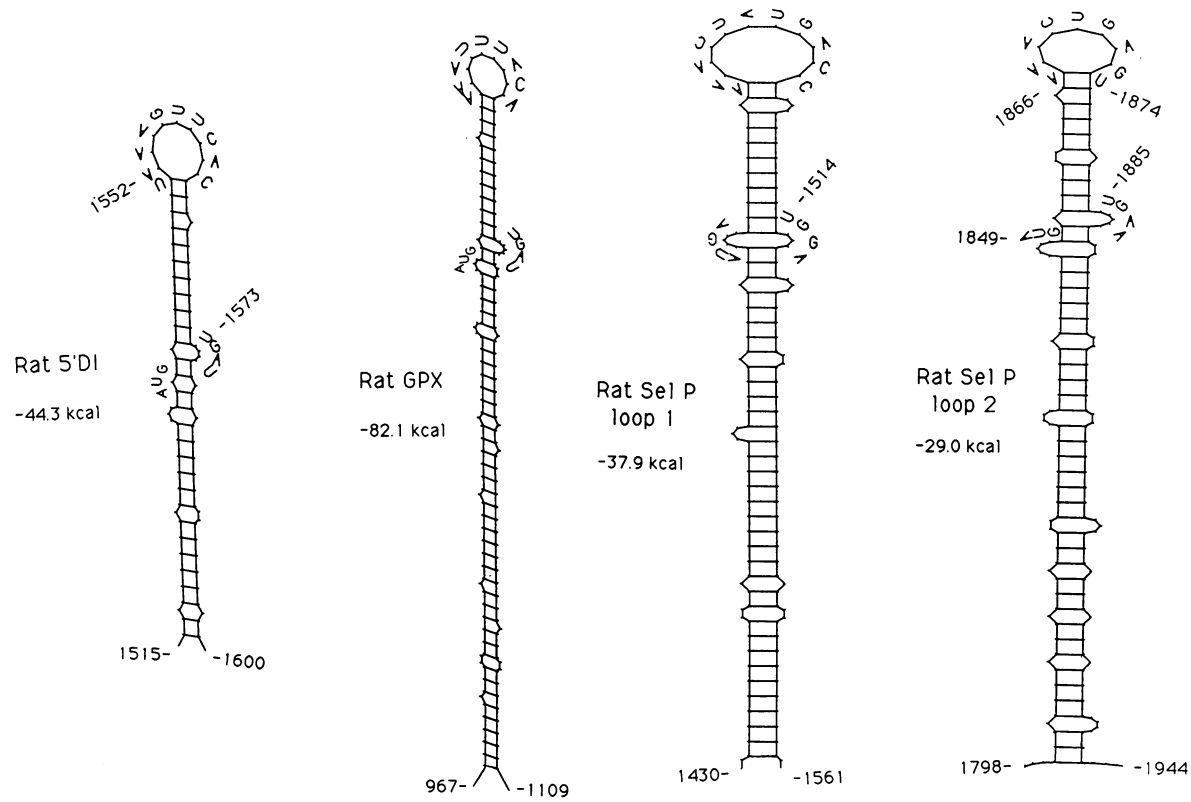

Fig. 1. Predicted secondary structures in SECIS elements from rat 5'DI, rat GPX and rat Sel P stem-loops 1 and 2. Structures were generated using the University of Wisconsin Genetics Computer Group Fold program (Zuker and Steigler, 1981).

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5'DI (1528) ATTTGTTTAT GATGG..CAC AG..TAAAGT .CACAC.GCT GTG.A...CT TGATT.....
  GPX (1009) .TT...C.AT GA.GGTGTT. CCTCTAAA.. T.C..GG... .A......CC TGAT.T....
SELP1 (1466) TTRC.TTRAT GA.GAAYAGA AACRTAAACT ATGACCTAGG GGTTT.CTGT TGGATARYT.
SELP2 (1841) ....RTCAAT GAYGGTTTAA TAGR.AAACY RARYCCTATR AA......CC TGA.CTCCTT

CONSENSUS  ......Y.AT GA.GR..... .....AAA.. ..... .....Y TGR.....
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Fig. 2. Conserved sequences in SECIS elements from rat and human 5'DI (Berry *et al.*, 1991a), rat, human, murine and bovine GPX (Berry *et al.*, 1991a) and rat and human Sel P stem-loops 1 and 2 (Hill *et al.*, 1993). Stem-loop sequences were compared using the Lineup program of the University of Wisconsin Genetics Computer Group. The position of the first base in each sequence from rat is indicated in parentheses.

identifies the following conserved features: three A residues in the loop, an unpaired AUGA in the 5′ arm of the stem and an unpaired UGR in the 3′ arm. In addition, the distance between these unpaired bases and the loops, 10–12 bases, is highly conserved. To assess the function of the putative Sel P SECIS elements, we used the rat 5'DI coding region as a reporter for translation of UGA as selenocysteine. Use of this system is based on our previous demonstration that expression of deiodinase activity from the wild-type 5'DI requires a functional SECIS element (Berry *et al.*, 1991a). We examined the two loops of Sel P in combination and individually for their ability to confer deiodinase expression. Surprisingly, a segment of Sel P 3′ UTR consisting of both loops and an additional ~600 nt produced nearly four-fold higher activity than the rat 5'DI SECIS element (Figure 3). Deletion of the last ~600 nucleotides decreased activity slightly. The first stem-loop alone was ~3-fold more active than the 5'DI SECIS element. The activity of the second Sel P stem-loop was comparable to that of the 5'DI, and deletion of the 8 bp loop from the second element eliminated function, as was previously found in the 5'DI and GPX elements (Berry *et al.*, 1991a).

Three conserved A residues in the SECIS loop are optimal for function

As the conservation in sequence and structure between SECIS elements from three different genes suggests that these particular features may be functionally important, we used site-directed mutagenesis to alter specific bases in the various elements. Mutagenesis of three conserved A residues to C residues in the rat 5'DI loop resulted in a >90% loss of activity, indicating a strong preference for one or more of the A residues (Table I). The As were next examined individually. Mutagenesis of the first A to G reduced the activity to ~45% of wild-type, indicating that this base contributes to function but is not essential. However, interpretation of this result is complicated by the potential for an additional base-pair between the G and the C opposite it at the bottom of the loop. Conversion of the second A to U produced an ~85% decrease in activity, identifying this base as more critical. Comparison of activity in the AUA and AUG mutants (Table I, 14.5% versus 0.7%) indicates that the third conserved A is also required for optimal activity. Additional substitutions at the second and third positions resulted in >95% loss of function.

Unpaired nucleotides in the stem are critical for SECIS function

Specific bases in the stems are conserved between the various selenoprotein 3′ UTRs, and some of these are predicted to be unpaired, forming 'bulges'. The rat and human 5'DI and GPX SECIS elements all contain a conserved, unpaired

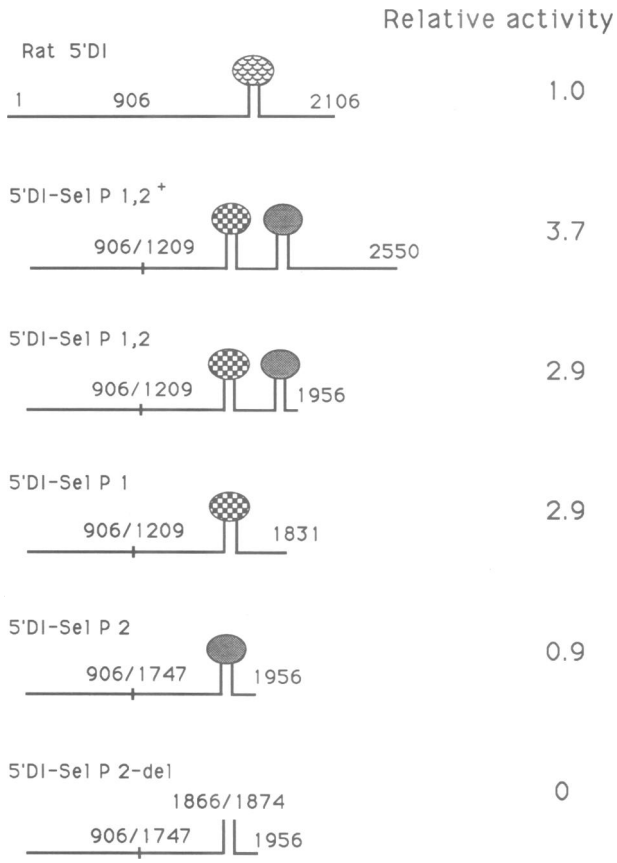

Fig. 3. Sel P contains two functional SECIS elements. The indicated sequences from the 3′ UTR region of the rat Sel P cDNA were cloned downstream of the rat 5′DI coding region. Deiodinase assays were performed as described in Materials and methods.

Table I. Mutagenesis of rat 5′DI SECIS element loop

Mutant	Activity (% w.t.)	Secondary structure effects
AAA (w.t.)	100	
CCC	7.9 ± 1.1	none
GGG	0	additional bp at top of stem
GAA	45.8 ± 5.3	additional bp at top of stem
AUA	14.5 ± 0.5	none
AUG	0.7 ± 0.14	none
ACU	2.6 ± 0.21	none
AGU	1.2 ± 0	none

Constructs contained nts 1–906 from the rat 5′DI linked to the rat 5′DI SECIS element with the indicated substitutions (nts 1552–1554), in CDM-8 vector. Deiodinase assays were performed as described in Materials and methods.

UGAU in the 3′ arm of the stem. The first Sel P SECIS element contains a UGG and the second element a UGA in analogous positions. Analysis of mutations of these unpaired bases shows that, in the rat 5′DI, the G and A are both required for function (Table II, compare UGA with UGU, and UGU with UUU). Similar analyses in the GPX stem-loop and the second Sel P stem-loop indicate that the same bases in these two SECIS elements are also essential. A conserved, unpaired AUGA is present in the 5′ arm of the stem in all of the SECIS elements, and substitutions at these bases in the second Sel P SECIS element also abolished expression, delineating their essential role.

Table II. Mutagenesis of 5′DI, GPX and Sel P SECIS unpaired bulges

	Nucleotides	Activity (% w.t.)
5′DI		
UGA (w.t.)	1573–1575	100
UGU	1573–1575	1.8 ± 0.28
UUU	1573–1575	0.11 ± 0.14
GPX		
UGA (w.t.)	1055–1057	100
UCU	1055–1057	2.6 ± 0.5
ACU	1055–1057	3.5 ± 1.2
Sel P loop 2		
UGA (w.t.)	1885–1887	100
UCU	1885–1887	0.4 ± 0.2
AUG (w.t.)	1849–1851	100
UAG	1849–1851	0
UAC	1849–1851	0

Constructs contained nucleotides 1–906 from the rat 5′DI linked to the SECIS elements from rat 5′DI, GPX or Sel P cDNAs with the indicated substitutions, in CDM-8 vector. Deiodinase assays were performed as described in Materials and methods.

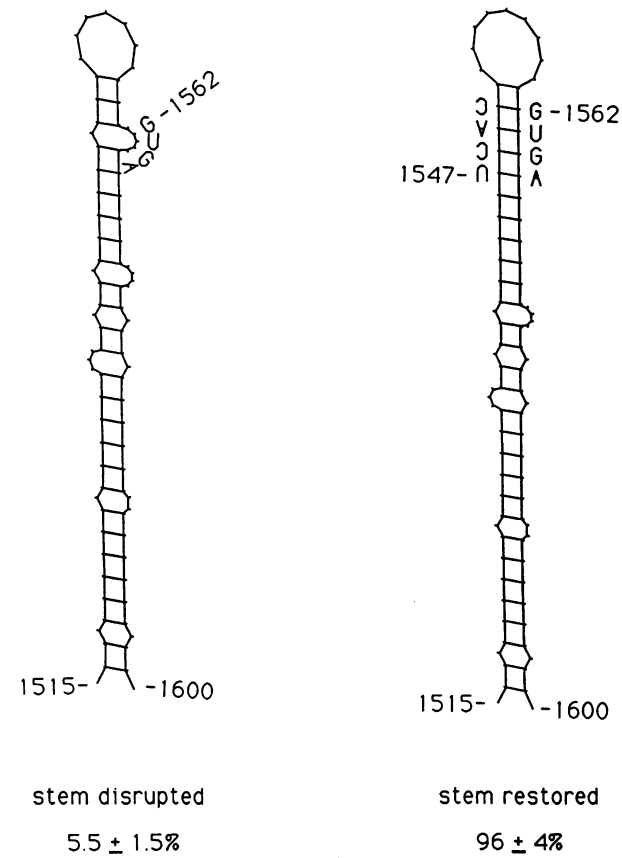

Fig. 4. Predicted secondary structural effects of stem mutations in rat 5′DI SECIS element. Sequences are shown for the mutated bases (wild-type shown in Figure 1). Structures were generated using the Fold program (Zuker and Steigler, 1981). Deiodinase activities are indicated, relative to the wild-type rat 5′DI SECIS element.

Secondary structural effects

We next examined the requirement for particular secondary structural features in the rat 5′DI SECIS element. Changing the sequence of five bases near the top of the stem (nts

1562–1566), such that complementarity for base-pairing is lost, results in loss of function (Figure 4). Compensating changes to the opposite side of the stem (nts 1547–1550), restoring potential for base-pairing, restored full activity, supporting the role of secondary structure, and not primary sequence, in this region. Mutation of a single base at the top of the stem (nt 1561, A to C), disrupting the top base-pair (U-A to U-C) and making the loop two nucleotides larger, resulted in an ~40% decrease in activity. A compensating change opposite this position (nt 1551, U to G), producing a G-C pair in place of the U-A, resulted in the same level of activity as the first mutant, indicating that the increase in loop size does not explain the decrease in activity. Finally, addition of a single base on the left arm

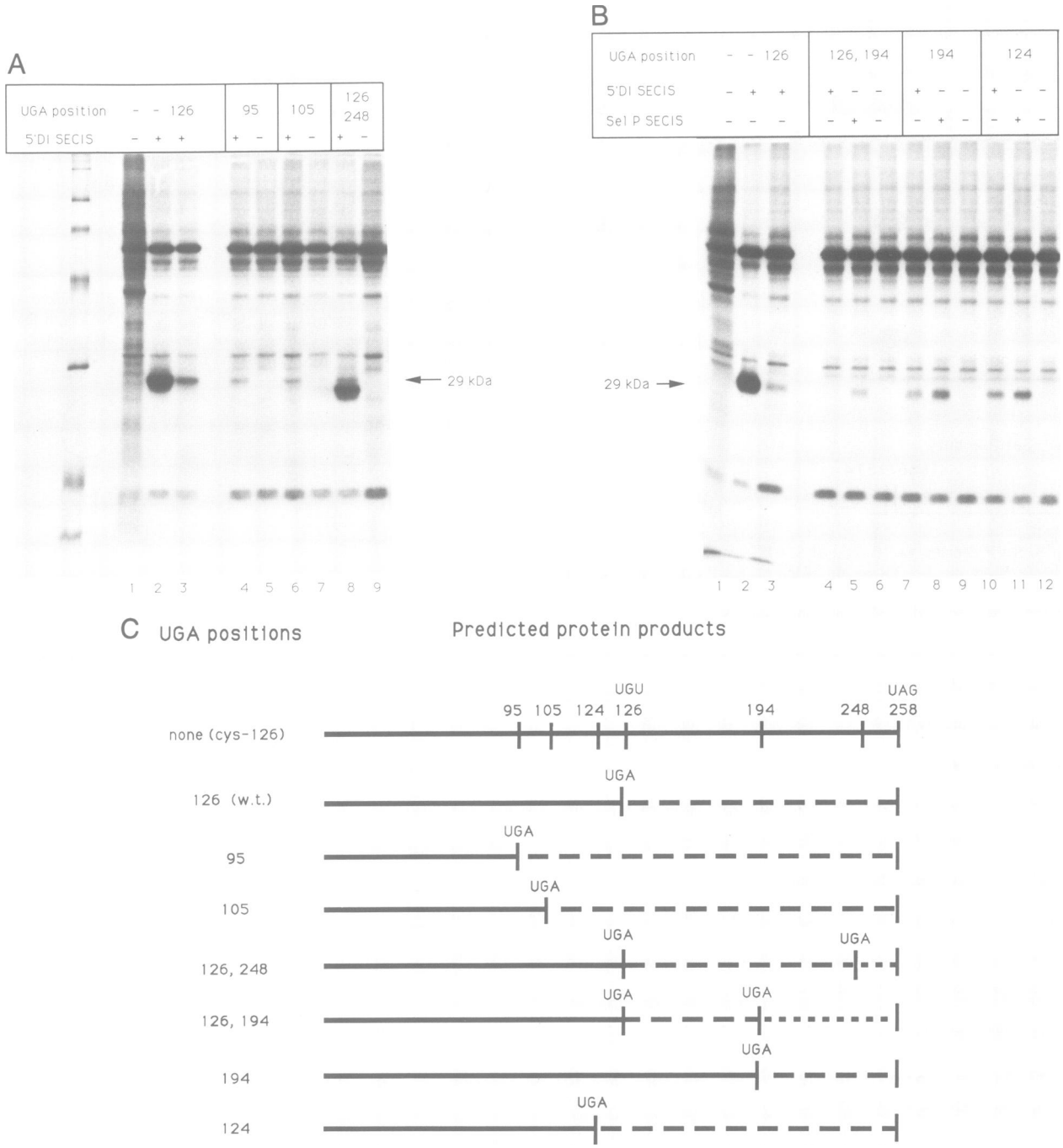

Fig. 5. Affinity labelling of 5'DI proteins produced by translation through mutant UGA codons. (**A**) Rat 5'DI constructs contain UGA codons at the indicated positions (Berry *et al.*, 1991b) and either the wild-type UGA or a cysteine codon in place of the wild-type UGA. Labelling with [^{125}I]BrAcT$_3$ was performed as described in Materials and methods. Lane 1, CDM-8 vector control; lane 2, 5'DI cysteine mutant at position 126; lane 3, wild-type 5'DI; for lanes 4–9, the presence or absence of SECIS elements and the positions of UGAs are indicated above the lanes. The far left lane contains ^{14}C-labelled protein standards of 200, 94, 68, 45, 30, 18 and 14 kDa. (**B**) The 3' UTR region consists of either that of the 5'DI, or both 3' UTR stem-loops of Sel P, or the 3' UTR of 5'DI with the SECIS region deleted. Lanes 1–3 as above; for lanes 4–12, the SECIS elements and positions of UGAs are indicated above the lanes. (**C**) Schematic representation of the UGA mutant constructs analyzed and the predicted protein products. Constructs are shown in order from top to bottom corresponding to the order from left to right in panels A and B. Solid lines represent efficient translation through regions without UGA codons. Dashed lines indicate decreased translational efficiency due to the presence of a single UGA codon. Dotted lines represent further reduction in efficiency beyond a second UGA codon.

of the 5'DI stem (between nts 1548 and 1549), opposite an unpaired base on the right arm, increasing the spacing between the bulges and the loop by one base-pair, resulted in an ~25% decrease in activity, indicating that the wild-type spacing may be optimal, but a slight increase is tolerable.

A specific UGA codon context is not required for selenocysteine incorporation in eukaryotes

We next examined the role of UGA codon environment or context on the efficiency of selenocysteine insertion in eukaryotes. As discussed above, selenocysteine incorporation into fDH in *Escherichia coli* requires that a specific stem-loop be located immediately adjacent to the UGA codon. To address this requirement in eukaryotes, site-directed mutagenesis was used to introduce UGA codons into the rat 5'DI coding sequence. In two constructs, a cysteine codon (codon 194 or 248) was converted to a UGA in the wild-type 5'DI, thus necessitating translation through two UGA codons. The other mutations were prepared in a 5'DI construct in which the wild-type UGA (codon 126) had been changed to a cysteine codon (Berry *et al.*, 1991c). In the background of this cysteine mutant, four additional cysteine codons (codons 95, 105, 124 and 194) were converted to UGA codons, producing four constructs with a cysteine codon in the wild-type position and a UGA in an 'out-of-context' position. Translation of the UGA codons was assessed by [^{125}I]$BrAcT_3$ affinity labelling of COS cell homogenates following transient expression. $BrAcT_3$ is a substrate analog of T_3 which covalently labels both the wild-type and Cys126 mutant 5'DI proteins (Berry *et al.*, 1992). All four constructs containing a single 'out-of-context' UGA codon produced $BrAcT_3$-labelled proteins of the same size as the wild-type enzyme (Figure 5A and B). These proteins were not detectable in extracts from cells transfected with vector alone (lanes 1) or in extracts from constructs which did not contain a SECIS element (Figure 5, panel A, lanes 5, 7 and 9, and panel B, lanes 6, 9 and 12). The UGA-248 mutant also produced a prominent $BrAcT_3$-labelled protein of the size expected from termination at the second UGA (Figure 5A), indicating that the truncated protein also binds substrate analog. UGA-terminated products from the other constructs are not seen with BrAcT3 labelling, presumably due to the inability of the truncated proteins to bind substrate. Because the Sel P SECIS region was more active than that of the 5'DI, this sequence (nts 1209–2550, containing both Sel P loops) was introduced into the 3' UTR region of three of the UGA mutants. The construct containing the wild-type UGA and a UGA-194 mutation expressed only a small amount of full-length protein when the 5'DI SECIS element was present (Figure 5B), and this increased with the Sel P SECIS element. A similar increase was observed with the two single UGA mutants tested (Figure 5B, compare lane 7 with 8 and lane 10 with 11), consistent with the results in Figure 3. Since the efficiency of translation of the wild-type 5'DI UGA codon in transfected cells is lower than that of the cysteine mutant (Berry *et al.*, 1992), the lighter band from cells transfected with the UGA-126,194 construct reflects the difficulty in translating two UGAs versus one.

Homogenates of cells transfected with these mutants were also assayed for expression of deiodinase activity. The nearly

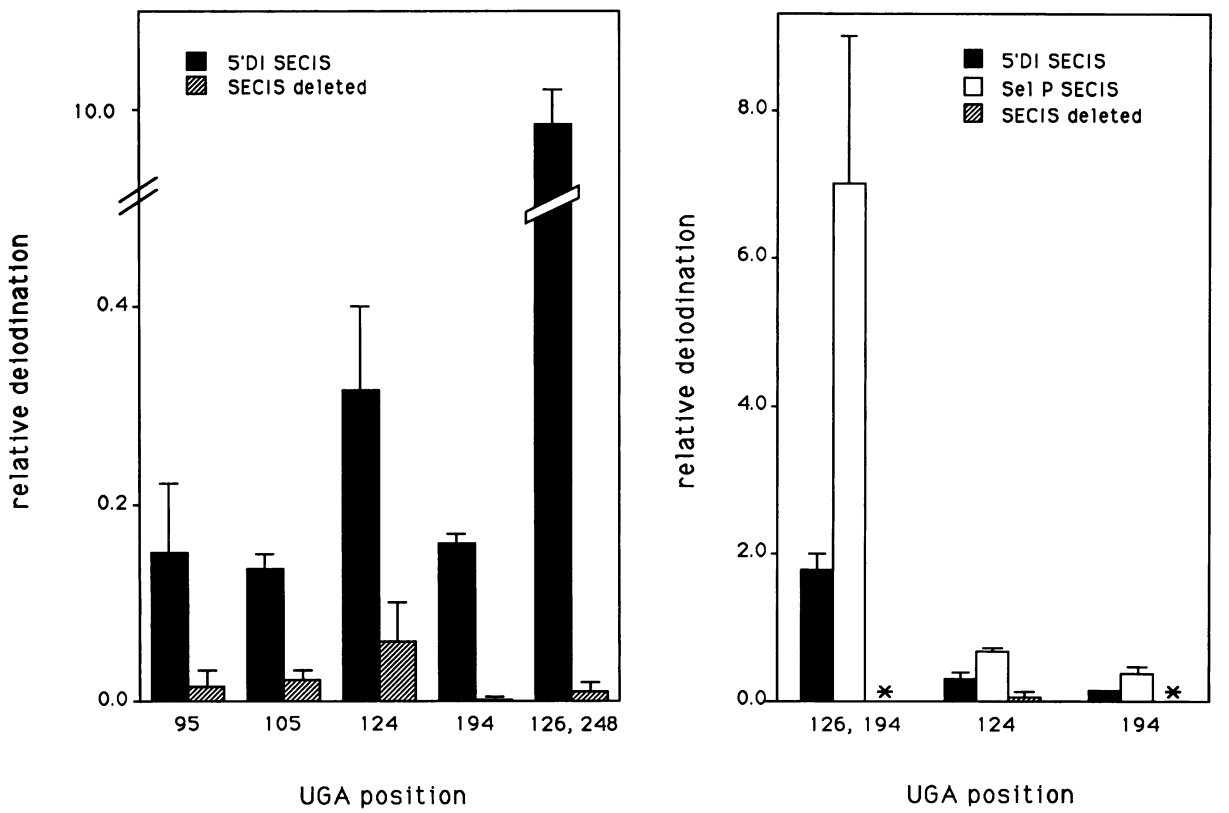

Fig. 6. 5'DI activity in out-of-context UGA mutants. Deiodinase activity was assayed in transfected cell sonicates as described in Materials and methods, and is expressed as deiodinase/hGH ratios. (**A**) Constructs contained the SECIS element from rat 5'DI (solid bars), or no SECIS element (hatched bars). (**B**) SECIS elements from rat 5'DI (solid bars), or Sel P (open bars) or no SECIS element (hatched bars). Asterisks indicate activity not different from background (vector transfected).

full-length 248 mutant produced activity comparable to wild-type, consistent with production of a fully active but truncated protein (Figure 6A). The activity produced by all other UGA mutants was lower that produced by the wild-type and cysteine mutant proteins. The UGA-194 mutant in the wild-type background (Figure 6B) was considerably more active (~13% of w.t.) than any of the UGA mutants in the cysteine background (~1–3% of w.t.), despite the fact that the efficiency of read-through of this mRNA is low (Figure 5B). This is consistent with our previous studies showing that the catalytic efficiency of the wild-type enzyme is about two orders of magnitude greater than that of the cysteine mutant (Berry *et al.*, 1992). For each 'out-of-context' UGA mutant, the deiodinase activity was significantly greater with the 5'DI SECIS present than it was for the SECIS deletion constructs (Figure 6A), and substitution of the Sel P SECIS element increased activity up to 4-fold compared with the 5'DI SECIS element (Figure 6B). The construct containing two UGAs and the Sel P SECIS element produced deiodinase activity at ~60% of the wild-type level. Thus, relatively efficient selenocysteine incorporation can be obtained with an 'out-of-context' UGA and a highly active SECIS element.

Effects of SECIS element position on function

Finally, to gain further understanding of the mechanism of SECIS element function, we examined the effects of introducing the rat 5'DI SECIS element in different positions relative to the coding region. In the wild-type rat 5'DI cDNA, the distance between the UGA codon and the middle of the stem-loop is ~1.2 kb. When the coding and SECIS regions were separated by inserting an additional 1.5 kb of 'spacer' DNA, increasing the distance to ~2.7 kb, expression of deiodinase activity was comparable to wild-type (Figure 7). The spacer sequence alone was inactive. We next examined the ability of SECIS elements to function when introduced 5' of the coding sequence, with the 3' SECIS element deleted. Interestingly, the 5'DI SECIS element conferred expression in the 5' position, but the efficiency was much lower than that of the wild-type 5'DI construct (Figure 7). The most surprising results were obtained when 5'DI or Sel P SECIS elements were cotransfected with the 5'DI coding sequence, but on separate plasmids. The SECIS elements conferred expression *in trans*, in a dose-dependent fashion (Figure 7), albeit with much lower efficiency than wild-type. Mutant SECIS elements *in trans* (5'DI del, SelP 2 del) were not functional. To prove that this was not due to recombination between plasmids containing coding and SECIS sequences, the corresponding RNAs were transcribed *in vitro* and injected into *Xenopus* oocytes, followed by assay of deiodinase activity in oocyte homogenates. Coinjection of the 5'DI coding and SECIS RNAs resulted in deiodination of 2.1% of total substrate, as compared with 0.6% with the coding and mutant SECIS RNAs, and 0.4% with the coding sequence alone, confirming the transfection results.

Discussion

The importance of RNA stem-loops, pseudoknots and other secondary structures in mediating biological functions has been documented in an increasing number of systems. These structures often exhibit functional requirements for specific primary sequences in loops and bulges, and for secondary structure in base-paired regions of stems, suggesting that proteins mediate recognition of these structures. Protein recognition of both base-paired regions and specific unpaired nucleotides play important roles in conferring specificity in tRNA synthetase recognition of cognate tRNAs (Rould *et al.*, 1991), regulation of ferritin and transferrin receptor expression by iron response element binding proteins (Bettany *et al.*, 1992), translational control of *E.coli* ribosomal protein synthesis (Cerretti *et al.*, 1988; Said *et al.*, 1988), and binding of the TAT to the TAR region of HIV RNAs (Roy *et al.*, 1990; Calnan *et al.*, 1991; Weeks and Crothers, 1991).

A recent review describes several examples of RNA

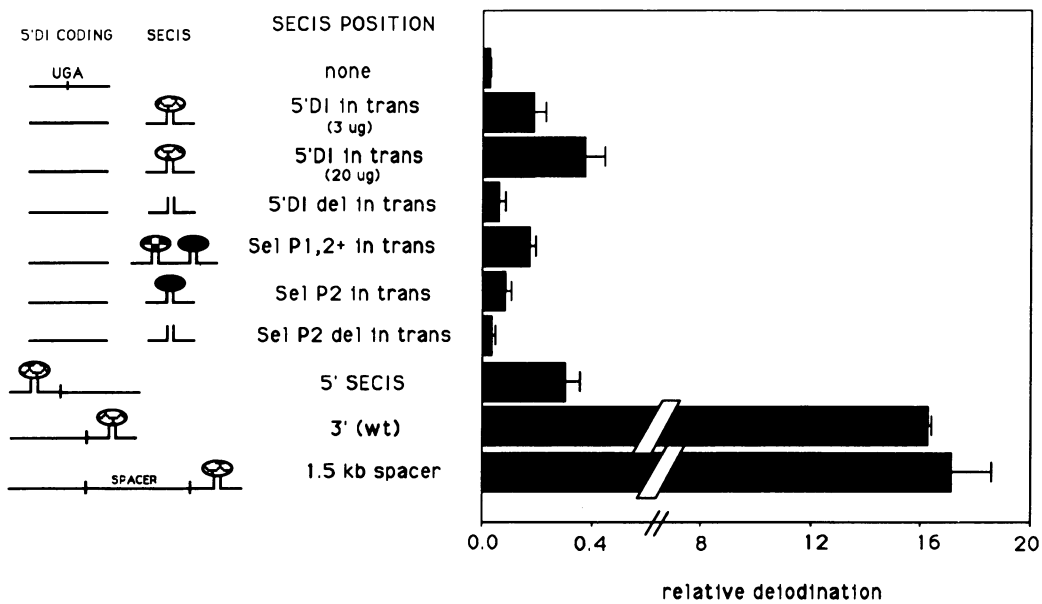

Fig. 7. Activity of SECIS elements at a distance and *in trans*. 5'DI and SelP SECIS sequences were assayed for ability to confer 5'DI expression when located 1.5 kb downstream or immediately upstream of the coding region, or cotransfected on a separate plasmid. All plasmids were transfected at 10 μg per 60 mm dish unless otherwise indicated. Deiodinase activity was assayed in transfected cell sonicates as described in Materials and methods.

structures which alter the reading of the genetic code, or 'recode' translation (Gesteland *et al.*, 1992). The studies reported herein describe the salient features of a system in which both primary sequence and secondary structure contribute to recoding. The requirement for several distinct features indicates that multiple contact sites are required between the SECIS element and the factor(s) mediating its function.

Examination of predicted secondary structures around the 5′DI and GPX UGA codons and in the coding region of Sel P identifies no common features in UGA codon context, suggesting that there is no stringent requirement for a particular environment for UGA codon translation. The results reported herein confirm this. Clearly, positions other than that of the wild-type UGA in the rat 5′DI can be recognized and translated as selenocysteine; the implications of this are discussed below. Nonetheless, the possibility exists that certain UGA codons are translated more efficiently than others, and perhaps ribosomal pausing occurs at some positions more than others. The results with the constructs containing two UGA codons show that one stem-loop is sufficient for translation of more than one UGA, and further, that translation of multiple UGA codons does not require a Sel P SECIS element, but can be directed by the 5′DI element.

These results establish an important difference in the mechanisms of prokaryotic and eukaryotic selenocysteine codon translation, namely the capacity of eukaryotic SECIS elements to confer this translation on any adjacent UGA codon. SECIS elements in 3′ UTRs relieve the necessity for stem-loop structures in the coding region, a requirement characteristic of prokaryotic selenoprotein mRNAs (Bock *et al.*, 1991; Heider *et al.*, 1992), thus permitting complete amino acid sequence flexibility in eukaryotic selenoproteins. We suggest a model whereby the stem-loops in the 3′ UTR regions interact with one or more as yet unidentified factors, possibly including a selenocysteyl-tRNA specific elongation factor analogous to the prokaryotic SEL B (Figure 8). The formation of a stem-loop/elongation factor/selenocysteyl-tRNA complex would then allow translation of UGA codons in the adjacent coding region, without a strict requirement for the UGA(s) to be in a particular environment. The efficiency of UGA codon translation according to this model would be expected to be low, and we have found this to be true (Berry *et al.*, 1992). Factors contributing to the low efficiency of the SECIS element in the 5′ position may include the stem-loop impeding progress of the advancing ribosomes or melting of base-pairing in the stem by the ribosomes. Possibly the SECIS element *in trans* functions by allowing accumulation of the stem-loop/elongation factor/tRNA complex in high enough concentrations such that it can interact with UGA codons, either by diffusion or by interaction of the complex with ribosomes. These studies were done in transiently transfected cells with high concentrations of both coding and SECIS DNA. Thus, while these *in vitro* effects are interesting and may be useful in studying the process of selenocysteine insertion, the *in vivo* efficiency is likely to be so low as to be insignificant to the cell. If this process occurred with any appreciable efficiency, it would certainly be detrimental to the cell, producing selenocysteine insertion where termination should occur.

Studies of selenoprotein synthesis at varying Se concentrations indicate a hierarchy, i.e. certain selenoproteins have priority on Se when it is limiting (Hill *et al.*, 1991; Gross *et al.*, 1992; Meinhold *et al.*, 1992). The studies described herein suggest that differences in efficiency of stem-loop function may allow the cellular translation machinery to express some selenoproteins selectively in preference over others, and in fact, the relative efficiencies of the SECIS elements in Sel P, 5′DI and GPX parallel their order of appearance upon selenium repletion in selenium-depleted animals (Hill *et al.*, 1991; Gross *et al.*, 1992; Meinhold *et al.*, 1992). Identification of Sel P SECIS elements with higher efficiency than the previously

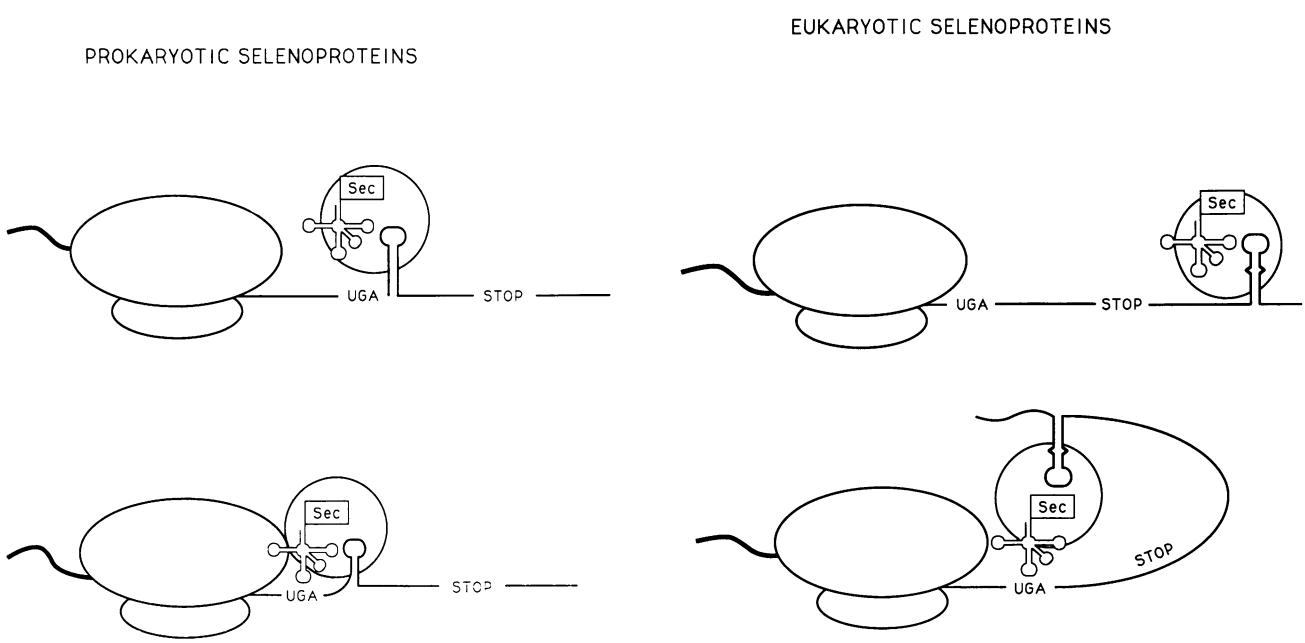

Fig. 8. Model for selenocysteine incorporation in prokaryotes and eukaryotes. The left panel (adapted from Heider *et al.*, 1992) shows interaction of prokaryotic Sel B (designated by the circles) with selenocysteyl-tRNA$^{(Ser)\ Sec}$, the stem-loop and the ribosome approaching the UGA codon. The right panel shows the putative eukaryotic Sel B homolog/SECIS element/selenocysteyl-tRNA$^{(Ser)\ Sec}$ complex. The eukaryotic model allows SECIS function with the element located at varying positions, in agreement with the data in Figure 7.

characterized elements, along with the UGA codon context results, provides the background for introduction of selenocysteine into other eukaryotic proteins. Taking advantage of this system and the differences in properties between selenocysteine and cysteine (Axley *et al.*, 1991; Berry *et al.*, 1991c, 1992) should allow advances to be made in mechanistic studies of sulfhydryl active site enzymes. Finally, the ability to introduce a heavy metal into a specific site in proteins could prove to be a powerful aid to three-dimensional structure studies.

Materials and methods

SECIS and UGA mutant constructs

The rat Sel P cDNA was generously provided by Drs Kristina Hill and Raymond Burk. For analysis of Sel P SECIS elements, the indicated regions of this cDNA were cloned into the CDM-8 vector (Aruffo and Seed, 1987) containing the first 906 nts of the rat 5'DI cDNA (coding region from nt 7 to nt 777). The rat GPX cDNA was kindly given by Dr Ye-Shih Ho. Nucleotides 922–1155 were cloned into the 5'DI-CDM vector for SECIS studies. Mutagenesis of SECIS elements was performed using either PCR methods (Higuchi, 1990) or the oligonucleotide-directed Altered Sites Mutagenesis System (Promega). The same methods were used to create UGA codons. All mutated regions were sequenced in their entirety to confirm that no other mutations were present. *Sma*I and *Pml*I sites on either side of the SECIS region in the rat 5'DI were used to delete the SECIS region in the UGA mutant constructs.

Transfections and deiodinase assays

COS-7 cells in 60 mm dishes were transfected with chimeric constructs in CDM-8 vector (10 μg per dish unless otherwise indicated) by $CaPO_4$ coprecipitation as described previously (Brent *et al.*, 1989). Cells were harvested 2 days after transfection, sonicated and incubated with [^{125}I]rT_3 (600 nM) for 30 min at 37°C. Release of $^{125}I^-$ was quantified as described previously (Berry *et al.*, 1991c). Activity was normalized to human growth hormone (hGH) in the medium produced from a cotransfected, constitutively expressed, hGH-encoding plasmid. All transfections and assays were in duplicate and all experiments were performed at least twice.

[^{125}I]BrAcT_3 labelling

Synthesis of [^{125}I]BrAcT_3 has been described in detail elsewhere (Berry *et al.*, 1992). Affinity labelling was performed with 100 μg transfected COS cell sonicate protein in a 50 μl reaction volume containing 0.05 μCi [^{125}I]BrAcT_3, 0.1 M potassium phosphate, pH 6.9, 1 mM EDTA and 20 mM DTT. Reactions were incubated for 10 min at 37°C and analyzed on denaturing 12.5% polyacrylamide gels.

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