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Eukaryotic Selenocysteine Incorporation Follows a Nonprocessive Mechanism That Competes with Translational Termination*

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The synthesis of eukaryotic selenoproteins involves the recoding of an internal UGA codon as a site for selenocysteine incorporation. This recoding event is directed by a selenocysteine insertion sequence in the 3′-untranslated region. Because UGA also functions as a signal for peptidyl-tRNA hydrolysis, we have investigated how the rates of translational termination and selenocysteine incorporation relate to *cis*-acting elements in the mRNA as well as to *trans*-acting factors in the cytoplasm. We used *cis*-elements from the phospholipid glutathione peroxidase gene as the basis for this work because of its relatively high efficiency of selenocysteine incorporation. The last two codons preceding the UGA were found to exert a far greater influence on selenocysteine incorporation than nucleotides downstream of it. The efficiency of selenocysteine incorporation was generally much less than 100% but could be partially enhanced by concomitant overexpression of the tRNA^{Sec} gene. The combination of two or three UGA codons in one reading frame led to a dramatic reduction in the yield of full-length protein. It is therefore unlikely that multiple incorporations of selenocysteine are processive with respect to the mode of action of the ribosomal complex binding to the UGA site. These observations are discussed in terms of the mechanism of selenoprotein synthesis and its ability to compete with termination at UGA codons.

Selenocysteine, the twenty-first amino acid (1, 2), is cotranslationally incorporated into a number of prokaryotic and eukaryotic proteins (3, 4). Although many of the basic mechanistic principles underlying this process in bacteria have been elucidated, the incorporation pathway in eukaryotic cells remains unknown. In all systems studied so far, the opal nonsense codon UGA is given an alternative coding status that allows it to encode selenocysteine. In *Escherichia coli*, this recoding depends on the presence of a stem-loop structure bearing specific sequences immediately 3′ of the UGA (5). Unlike its prokaryotic counterpart, the eukaryotic element is located in the 3′-untranslated region (3′-UTR; Ref. 6),[1] and is capable of acting at distances greater than 5000 nucleotides (7, 8). This element has been called the selenocysteine insertion sequence (SECIS). The highly conserved sequences in the SECIS element are: AAA in or near the apical loop, GA on the 3′ side of the stem, and AUGA on the 5′ side of the stem. A particular focus of attention has recently been the sequence region including the GA and AUGA elements, which is thought to form a non-Watson-Crick base paired duplex structure (9, 10).

The recoding of UGA depends on a number of specific *trans*-acting components. In bacteria, a special type of seryl-tRNA (tRNA^{Sec}; SelC) decodes the opal codon as selenocysteine with the assistance of a dedicated EF-Tu-like factor called SelB (1, 2, 11, 12). The synthesis of selenocysteyl-tRNA^{Sec} from seryl-tRNA^{Sec} requires three further proteins (SelA, SelC, and SelD; Refs. 13–15). The greatest stumbling block to elucidating the mechanism of selenocysteine incorporation in eukaryotes has been uncertainty as to the nature of the eukaryotic equivalent(s) to SelB. There have been reports of at least four different SECIS-binding proteins (16–20). So far, a 120-kDa protein seems to be the most likely candidate for binding specifically to SECIS elements (20), but further work will be needed to determine the other binding partners of this factor and its role in the selenocysteine incorporation process. Most importantly, there is evidence that the 120-kDa protein forms a complex with other proteins, which may mean that the identified SECIS-binding factor is only part of a multicomponent machinery. One potential component of such a complex could be the selenocysteyl-tRNA^{Sec}-protecting factor described by Yamada and colleagues (21).

An important unresolved question is to what extent the synthesis of polypeptide chains beyond an internal UGA codon by virtue of selenocysteine incorporation may be substoichiometric. This would explain why the substitution of a cysteine codon (UGU or UGC) at the site of a UGA results in greatly enhanced levels of polypeptide synthesis (22–24). At the same time, it is essential to consider the implications of UGA recoding for the termination process on the ribosome. It has been suggested that unless the distance between the UGA and the SECIS element is suboptimal, termination will be suppressed (3). However, there has been no systematic study to date of the relationship between translation termination and selenocysteine incorporation. In the present work we examine the properties of the UGA site that can influence both the efficiency with which selenocysteine is incorporated and the rate of polypeptide chain termination. There is a further aspect of selenoprotein synthesis that has previously remained unexplained. Genes such as those encoding selenoprotein P (25) and human type 2 iodothyronine deiodinase (8) contain multiple UGA codons. This raises the question as to how selenocysteine

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[1] The abbreviations used are: UTR, untranslated region; SECIS, selenocysteine insertion sequence; PCR, polymerase chain reaction.

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TABLE I
Oligodeoxyribonucleotides used in this work

TGA codons, TGC codons, and +4 positions are underlined. The complementary oligonucleotide pairs are designated A and B, respectively.

1. PHGPXSL-A	5′-T CGA GTG GCC TCT CAA TGA GGC AAG ACG GAG GTA AAC TAC ACT CAG CTG GTC GAC CTG CAC GCC CGA
	TAT GCT GAG TGT GGT TTA CGG ATT CTG G-3′
2. PHGPXSL-B	5′-GA TCC CAG AAT CCG TAA ACC ACA CTC AGC ATA TCG GGC GTG CAG GTC GAC CAG CTG AGT GTA GTT TAC
	CTC CGT CTT GCC TCA TTG AGA GGC CAC-3′
3. PHGP-A	5′-C CCT CGA GTG GCC TCT CAA TGA AGC AAG ACG GAG GTA AAC-3′
4. PHGP-C	5′-C CCT CGA GTG GCC TCT CAA TGA CGC AAG ACG GAG GTA AAC-3′
5. PHGP-T	5′-C CCT CGA GTG GCC TCT CAA TGA TGC AAG ACG GAG GTA AAC-3′
6. PHGP-Xho	5′-C CCT CGA GTG GCC TCT CAA TGC GGC AAG ACG GAG GTA AAC-3′
7. PHGP-Bam	5′-CG GGA TCC CAG AAT CCG TAA ACC ACA CTC AGC ATA TCG G-3′
8. PHGP-12A	5′-T CGA GTG GCC GAC CGA TGA GGC AAG ACG GAG GTA AAC TAC ACT CAG CTG G-3′
9. PHGP-12B	5′-TC GAC CAG CTG AGT GTA GTT TAC CTC CGT CTT GCC TCA TCG GTC GGC CAC-3′
10. PHGP-LOA	5′-TCG AGT GGC CTC TCA ATG AGG CAA GAC GGA AGT TAA CTA TAC TCA GCT GG-3′
11. PHGP-LOB	5′-TCG ACC AGC TGA GTA TAG TTA ACT TCC GTC TTG CCT CAT TGA GAG GCC AC-3′
12. SelP 12XA	5′-GA TCT TGA TGC TGC CAT TGT CGA CAT CTG ATA TTT GAA AAA ACA GGG TCT GCA ATC ACC TGA TTA G-3′
13. SelP 12XB	5′-GA TCC TAA TCA GGT GAT TGC AGA CCC TGT TTT TTC AAA TAT CAG ATG TCG ACA ATG GCA GCA TCA A-3′
14. Sel P 1XA	5′-GA TCT TGA TGC TGC CAT TGT CGA CAT CTG ATA TTT GAA AAA ACA GGG TCT GCA ATC ACC TGC TTA G-3′
15. SelP 1XB	5′-GA TCC TAA GCA GGT GAT TGC AGA CCC TGT TTT TTC AAA TAT CAG ATG TCG ACA ATG GCA GCA TCA A-3′
16. SelP 2XA	5′-GA TCT TGC TGC TGC CAT CTG CGA CAT CTG ATA TTT GAA AAA ACA GGG TCT GCA ATC ACC TGA TTA G-3′
17. SelP 2XB	5′-GA TCC TAA TCA GGT GAT TGC AGA CCC TGT TTT TTC AAA TAT CAG ATG TCG ACA ATG GCA GCA GCA A-3′
18. SelP 12CA	5′-GA TCT TGC TGC TGC CAT TGT CGA CAT CTG ATA TTT GAA AAA ACA GGG TCT GCA ATC ACC TGC TTA G-3′
19. SelP 12CB	5′-GA TCC TAA GCA GGT GAT TGC AGA CCC TGT TTT TTC AAA TAT CAG ATG TCG ACA ATG GCA GCA GCA A-3′

can be repeatedly inserted at up to 10 positions within the same polypeptide. In the following work, we examine whether processivity is likely to be the molecular basis for this phenomenon.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—All DNA manipulations were performed according to standard protocols (26). Synthetic oligonucleotides used in plasmid constructions are listed in Table I. To construct the master plasmids pH8A and pH9A, the oligonucleotide pair (PHGPXL-A and PHGPXSL-B) containing the in-frame TGA codon of the phospholipid hydroperoxide glutathione peroxidase gene (nucleotides 165–255) of *S. scrofa* (GenBank™ accession number X76009) was subcloned between the *Bam*HI and *Sal*I sites of the plasmids pBLUGA and pBPHGPx3U (23). PBPHGPx3U contains the PHGPx SECIS element in its 3′-UTR. To mutate the TGA codon to TGC, PCR was carried out using the primers PHGP-Xho and PHGP-Bam, and the resulting PCR fragment was inserted between the *Sal*I and *Bam*HI sites of the plasmids pBLUGA and pBPHGPx3U. The resulting plasmids were designated pH8B and pH9B. To mutate the fourth base G to A, C, and T, several PCRs were carried out using the forward primers PHGP-A, PHGP-C, and PHGP-T combined with PHGP-Bam as a reverse primer, respectively. The resulting PCR fragments were inserted between the *Sal*I and *Bam*HI sites of the plasmids pBPLUGA and pBPHGPx3U. The resulting plasmids were designated pT11–pT16. The nature of the fourth base and the presence or absence of the PHGPx SECIS element are indicated in Table II.

To mutate the penultimate codons positioned at −1 and −2 relative to the TGA codon, the oligonucleotide pair (PHGPXL-A and PHGPXL-B) was digested with *Sal*I and then subcloned between the *Bam*HI and *Sal*I sites of the plasmid pBPHGPx3U, generating pT5. An oligonucleotide pair (PHGP-1–2 A/B) harboring a region of the PHGPx gene (the amino acids serine and glutamine at positions 44 and 45 were changed to aspartic acid and arginine, respectively) was subcloned in the *Sal*I site of the plasmid pT5. To destabilize the predicted stem-loop located downstream of the TGA codon, an oligonucleotide pair (PHGP-LOA and PHGP-LOB) containing mutations in the stem-loop was subcloned into the *Sal*I site of the plasmid pT5.

To investigate selenocysteine incorporation at multiple sites, additional in-frame TGA codons were inserted in the *lacZ::luc* reporter system. The oligonucleotide pairs SelP1X-A/B, SelP2X-A/B, SelP12X-A/B, and SelP12C-A/B containing the selenoprotein P gene of *H. sapiens* (GenBank™ accession number Z11793, amino acids 300–318; the in-frame TGA codons are located at positions 300 and 318) were subcloned in the *Bam*HI site of the plasmids pH8A, pH8B, pH9A, and pH9B. The oligonucleotide pairs contain TGA and TGC codons in various combinations so that the constructs contain one, two, or three TGA (or TGC) codons. The resulting plasmids were designated pT18–pT33. The plasmids are listed in Table II.

Xenopus $tRNA^{Sec}$ (the SelC equivalent) and the SelD gene (SelD4-CDM8) were provided by Dr. Alain Krol (27) and Dr. Marla Berry (28), respectively. The *Xenopus* release factor genes were supplied already cloned in the expression plasmids pCYM1–11/XSUP35 (29) and pX-LCL1 (30) by Prof. Michel Philippe (CNRS, Rennes, France).

TABLE II
The plasmid constructs

The table indicates whether cysteine (O) or selenocysteine (I) is encoded, changes in the +4 position (underlined), changes in codons −2 and −1 (DR), mutations in the downstream stem-loop structure (SL), and the presence (+) or absence (−) of the SECIS element.

Name of the constructs	Incorporation site (s)	Fourth base (underlined)	SECIS	Ref.
pH8B	O	TGCG	-	This work
pH8A	I	TGAG	-	This work
pT11	I	TGAA	-	This work
pT12	I	TGAC	-	This work
pT13	I	TGAT	-	This work
pH9B	O	TGCG	+	This work
pH9A	I	TGAG	+	This work
pT14	I	TGAA	+	This work
pT15	I	TGAC	+	This work
pT16	I	TGAT	+	This work
pT25	OOO	TGCG, TGCT, TGCT	-	This work
pT21	IOO	TGAG, TGCT, TGCT	-	This work
pT22	OIO	TGCG, TGAT, TGCT	-	This work
pT23	OOI	TGCG, TGCT, TGAT	-	This work
pT18	IIO	TGAG, TGAT, TGCT	-	This work
pT24	OII	TGCG, TGAT, TGAT	-	This work
pT19	IOI	TGAG, TGCT, TGAT	-	This work
pT20	III	TGAG, TGAT, TGAT	-	This work
pT29	IOO	TGAG, TGCT, TGCT	+	This work
pT30	OIO	TGCG, TGAT, TGCT	+	This work
pT31	OOI	TGCG, TGCT, TGAT	+	This work
pT26	IIO	TGAG, TGAT, TGCT	+	This work
pT32	OII	TGCG, TGAT, TGAT	+	This work
pT27	IOI	TGAG, TGCT, TGAT	+	This work
pT28	III	TGAG, TGAT, TGAT	+	This work
pT33	OOO	TGCG, TGCT, TGCT	+	This work
pBPLUGA	I	TGAG	-	Ref. 24
pBDI	I	TGAG	+	Ref. 24
pBPHGPx	I	TGAG	+	Ref. 24
pBPHGP3′UTR	I	TGAG	+	Ref. 24
PT9	DRI	TGAG	+	This work
PT10	ISL	TGAG	+	This work

Cell Culture, Gene Transfer, and Enzyme Assays—BHK-21 cells (baby hamster kidney cells; ATCC CC110) were cultivated as described elsewhere (24). Transient transfections were performed using Superfect transfection reagent (Qiagen) following the manufacturer's protocol. β-Galactosidase and luciferase activities were measured according to the protocol described previously (24). Selenium supplementation, where used, was achieved by adding $Na_2Se_2O_3$ to give a final concentration of 5 $\mu g\ l^{-1}$.

RNA Isolation and Reverse Transcriptase-PCR—Isolation of total RNA and reverse transcriptase-PCR were performed using TRIzol® reagent and Superscript™ (Life Technologies), respectively, following the manufacturer's protocols. PCR was performed using primers against the *lacZ::luc* mRNA and against actin mRNA (as a reference)

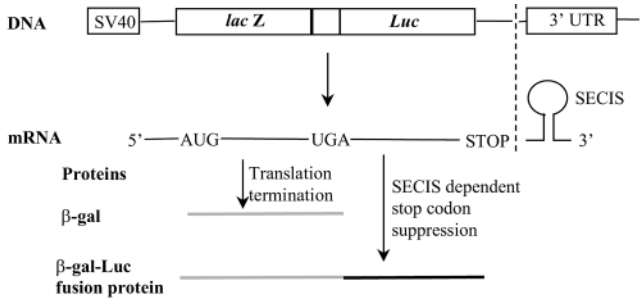


FIG. 1. **The reporter system used in this work.** This was based on the master construct described previously (24). The *lacZ* and *Luc* reading frames are joined by a spacer region that in the current work comprises segments of naturally occurring selenoprotein genes, as indicated in the later figures in this paper. In the absence of a SECIS element, termination at a UGA within the spacer yields a C-terminally extended version of β-galactosidase. Suppression of the UGA mediated by a SECIS element leads to synthesis of a β-gal::Luc fusion protein with both enzyme activities.

for 22, 26, 32, and 36 cycles.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—SDS-polyacrylamide gel electrophoresis was carried out according to the protocol described by Laemmli (31). Western blotting was performed using a semi-dry method (32) using cell extracts prepared according to an earlier protocoll (24).

Determination of Selenocysteine Incorporation and Translation Termination—The reporter system used in this work is based on the genes encoding β-galactosidase and luciferase, which are fused in-frame via a selenoprotein gene sequence including TGA stop codon(s) (24). Upon transfection of the respective plasmids into BHK-21 cell lines, the DNA is transcribed under control of the SV40 promoter, and translation leads to the synthesis of reporter enzymes. Translation terminates at the internal in-frame UGA stop codon, which leads to the synthesis of β-galactosidase enzyme. The presence of a SECIS element in the 3′-UTR suppresses the UGA stop codon by directing recoding for selenocysteine, which leads to the production of the β-galactosidase-luciferase fusion protein. Calculation of either termination or suppression efficiency is based on the parallel transfection of a plasmid which contains TGC (cysteine) instead of TGA. The ratio of enzymatic activities of the cells transfected with this plasmid was taken as the reference value (set at 100%). In the investigation of the influence of the +4 position on selenocysteine incorporation, the value obtained with G was taken as the 100% reference value.

RESULTS

The Environment of the UGA—The first stage of our investigation of the factors influencing selenocysteine incorporation efficiency in mammalian selenoproteins focused on the role of the immediate environment of the UGA site. It was our intention to obtain information about the possible upper limits of incorporation efficiency. We therefore chose the pig heart phospholipid hydroperoxide glutathione peroxidase (PHGPx; Ref. 33) gene as a starting point, because previous work had shown that the SECIS of this gene supported a higher incorporation efficiency than the other SECIS elements tested (24). We used a fusion reporter system (Ref. 24 and Fig. 1) to study the influence of the UGA environment. In using this system, we compare the suppression of termination at the central UGA in the presence and in the absence of the SECIS element. In this way, we can determine the recoding that is strictly related to a SECIS-dependent pathway, which reflects selenocysteine incorporation. The reporter gene system is expressed from the SV40 promoter *in vivo* after transfection of mammalian cells. Translation can either terminate at the C terminus of *lacZ* or, upon suppression of the nonsense function of the UGA codon, continue through the linker sequence to generate a fusion protein of β-galactosidase and luciferase (Fig. 1). Because we found that supplementation of the culture medium with Na_2SeO_3 had no effect on the results obtained with this reporter system (data not shown), all the experiments described

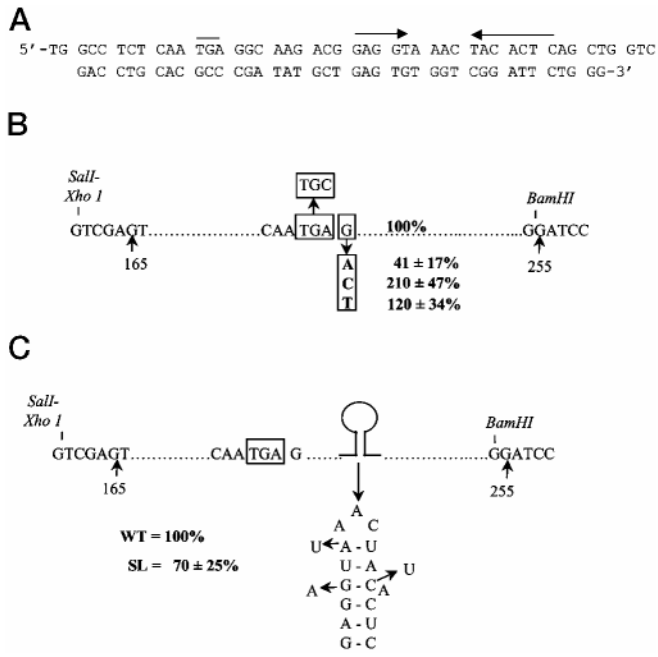
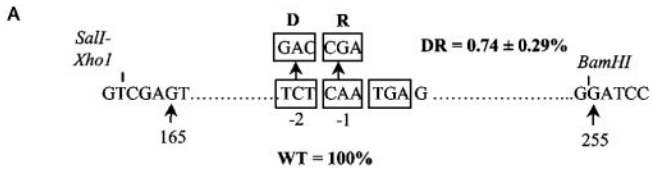


FIG. 2. **The influence of position +4 relative to the UGA codon on selenocysteine incorporation.** *A*, a segment of the porcine PHGPx gene (GenBank™ accession number X76009) containing nucleotides 165–255 was used as the site of selenocysteine incorporation between *lacZ* and *Luc* (compare Fig. 1). The TGA codon is *overlined*. The *arrows* indicate nucleotides that could form a putative stem-loop structure (see *panel C*). *B*, the above region was inserted between the *Bam*HI and *Sal*I sites of plasmids pBLUGA and pBPHGPx3′ U (Ref. 24, Fig. 1, and Table I). The fourth base was varied as shown. The TGA codon was also changed to TGC to generate cysteine-encoding controls. The efficiency of SECIS-dependent stop codon suppression was determined by measuring the activities of luciferase and β-galactosidase in transient transfection experiments of BHK-21 cells using plasmids without and with the PHGPx SECIS element. The luciferase activity was normalized to the activity of β-galactosidase using as a reference the efficiency obtained with cells that contained a TGC control construct. Each SECIS-dependent relative efficiency was then corrected by subtracting the corresponding SECIS-independent stop codon suppression efficiency. The values given here are expressed as percentages of the value obtained with the plasmid containing a guanine nucleotide in the +4 base position. The relative efficiencies represent mean values (± standard deviations), each of which was obtained from at least six different transfection experiments. All transfection experiments were carried out in duplicate. *C*, the effect of predicted downstream secondary structure on selenocysteine incorporation. To destabilize the putative downstream stem-loop, the nucleotides G, A, and C were changed to A, U, and U, respectively. The relative values obtained for the wild type (*WL*) and modified (*SL*) versions of the putative stem-loop structures are indicated.

in this paper were performed in the absence of a selenium supplement.

We examined whether the activity of the PHGPx UGA, like its counterpart in the type 1 deiodinase gene (34), is sensitive to the downstream context. The results confirmed that SECIS-dependent incorporation is increased when the fourth base is a pyrimidine (Fig. 2*B*). This effect is therefore common to different UGA contexts and SECIS elements. We wished to compare this result with the effects of changes in other features of the UGA context that have not previously been investigated. The first of these is the potential influence of mRNA structure downstream of the UGA. For example, a predicted stem-loop structure could potentially be formed 9 nucleotides downstream of the UGA in the PHGPx gene (Fig. 2*C*). It is known that downstream structure plays a key role in the selenocysteine incorporation process in *E. coli* (5). Could it also at least modulate the efficiency of eukaryotic selenocysteine incorporation, perhaps by virtue of its ability to cause ribosomes to pause



B

Accession numbers

	DRX
U43285	SWRLTSFSGMKG**X**GCKVPQETLLKLLEG
L41731	HRPLVLNFGSCT**X**PPFMARLQAYQRLAA
U11762	NRPLVLNFGSCT**X**PSFLFKFDQFKRLIE
AF093774	ERPLVVNFGSAT**X**PPFTSQLPAFRKLVE
U67890	MALAVRVVYCGA**X**GYKPKYLQLKEKLEH
X13709	RGKVLLINVASL**X**GTTVRDYTQMNELQR
X76009	GYVCIVTNVASQ**X**GKTEVNYTQLVDLHA
X99807	GKVTVVALLQAS**X**YLCLLQASRLEDLRI

FIG. 3. *A*, the amino acids at positions −2 and −1 were changed to aspartic acid and arginine, respectively. The effect of mutations (*DR*) in the penultimate and final codons on selenocysteine incorporation in transient transfection experiments is evident in the comparative Luc/β-gal values shown next to the diagram (wild type set to 100%). *B*, analysis of the amino acid context of selenoprotein genes. The amino acids aspartic acid and arginine, which promote efficient termination, are not encoded at the −2 and −1 positions of any known selenoprotein genes. *U43285*, mouse selenophosphate synthetase 2; *L417321*, *Rana catesbeiana* 5′ deiodinase type III; *U11762*, *Canis familiaris* type I iodothyronine deiodinase; *AF093774*, human type 2 iodothyronine deiodinase; *U67890*, mouse selenoprotein W; *X13709*, human glutathione peroxidase; *X76009*, pig phospholipid hydroperoxide glutathione peroxidase; *X99807*, mouse selenoprotein P.

longer at the UGA? We investigated whether mutating the putative stem-loop to destabilize it would affect selenocysteine incorporation (Fig. 2*C*). Any effect is evidently marginal, thus indicating that downstream secondary structure is unlikely to be involved in eukaryotic selenocysteine incorporation. We conclude that the nature of the nucleotide at the +4 position relative to the UGA is likely to be the most relevant feature of the downstream region in terms of determination of the rate of selenocysteine incorporation.

In stark contrast to this latter result, we found that changes in the codons immediately upstream of the UGA can markedly affect selenocysteine incorporation efficiency (Fig. 3). We replaced the penultimate and final amino acid encoding triplets preceding the UGA in PHGPx with two codons that belong to a group of termination-promoting codons (Fig. 3*A* and Ref. 35). The impact on selenocysteine incorporation was dramatic. We also observed that the combination of amino acids substituted in these experiments is avoided in all of the known UGA gene contexts found in selenoprotein genes (Fig. 3*B*). We conclude from this result that the selenocysteine incorporation rate of the PHGPx UGA is not normally restricted by the presence of 5′ neighbor codons that promote strong termination. Indeed, overall we were not able to identify any *cis*-acting elements within the open reading frame that could markedly improve selenocysteine incorporation efficiency at the PHGPx UGA.

Modulation by Trans-acting Factors—We investigated whether the use of a reporter system introduced by transient transfection might lead to partial saturation of the selenocysteine incorporation machinery, thus potentially restricting the maximum attainable efficiency of selenocysteine incorporation. The results of earlier studies were not conclusive. One group found that co-transfection of $tRNA^{Sec}$ (SelC) enhances selenocysteine incorporation in transiently transfected human embryonic kidney cells (23), whereas another group observed no increase in the synthesis of selenoproteins as a consequence of $tRNA^{Sec}$ overexpression in Chinese hamster ovary cells (36). To address this question in our reporter system, we examined whether the co-transfection of a SelC expression construct would affect the incorporation efficiencies. We found that the incorporation efficiency at UGAG or UGAC was increased by up to approximately 2-fold (Fig. 4). In further experiments, we co-transfected both SelC and SelD. The addition of the latter gene did not enhance the incorporation efficiency any further. In fact, the co-transfection of both SelC and SelD yielded a somewhat smaller enhancement in the incorporation efficiency. Overall, therefore, some increase in selenocysteine incorporation efficiency was supported by an enlargement in the size of the cellular $tRNA^{Sec}$ pool.

There is some ambiguity about the dual role of internal UGA codons in selenoprotein genes, in that estimates of the efficiency of selenocysteine incorporation have varied from a few percent of the polypeptide chains reaching the UGA being extended (23) to the suggestion that this process fully suppresses termination (3). Generally, the absolute efficiencies of selenocysteine incorporation in the known selenoproteins remain unknown quantities. Clearly, if termination and selenocysteine incorporation can both take place at the same UGA, these two processes may effectively proceed competitively. One possible way of testing this model is to examine whether increasing the levels of the translation release factors leads to suppression of selenocysteine incorporation. To perform this experiment, we co-transfected individual reporter constructs together with expression constructs bearing the genes encoding eRF1 and eRF3 (29, 30). Comparison with the control construct pBLUGA revealed that there was little specific repression of selenocysteine incorporation associated with the overproduction of the eRFs (Fig. 4*C*).

Permutations of Multiple UGA Codons—Further insight into the mechanism of selenocysteine incorporation can be obtained by studying how the system deals with open reading frames containing more than one UGA. Three sites were used for the insertion of either UGA or UGC (encoding cysteine; Fig. 5*A*). One of these was the original PHGPx site used in the earlier experiments (Fig. 2*A*), although the other two were excised from the SelP gene (nucleotides 934–990) and fused on to the 3′ end of the PHGPx gene segment. It was observed that the incorporation level differed for the respective sites used in this experiment (I00, 0I0, and 00I, Fig. 5*B*). The results described earlier in this paper indicate that this variation can be explained at least partially in terms of differences in the environment of each UGA. For example, the distinct identities of the final codon pairs immediately 5′ of each UGA may contribute to the differences in incorporation efficiency. This phenomenon was not investigated further because it is not of immediate relevance to the main objective of the multiple UGA experiments.

More strikingly, combining the individual UGAs in different permutations in a single reading frame resulted in very marked reductions in SECIS-dependent suppression of termination (Fig. 5*B*). The combination of UGAs at all three sites resulted in particularly low yields of luciferase activity. This effect was also evident in the amounts of β-galactosidase::luciferase protein detectable in cell extracts by means of Western blotting (Fig. 5*C*). The sharp reduction in the abundance of the full-length protein upon the introduction of more than one UGA into the reporter system is clearly apparent. Two further types of result indicate that this is due to alterations in the overall efficiency of selenocysteine incorporation rather than changes in the fate of the mRNA. First, we found that the levels of β-galactosidase encoded by these con-

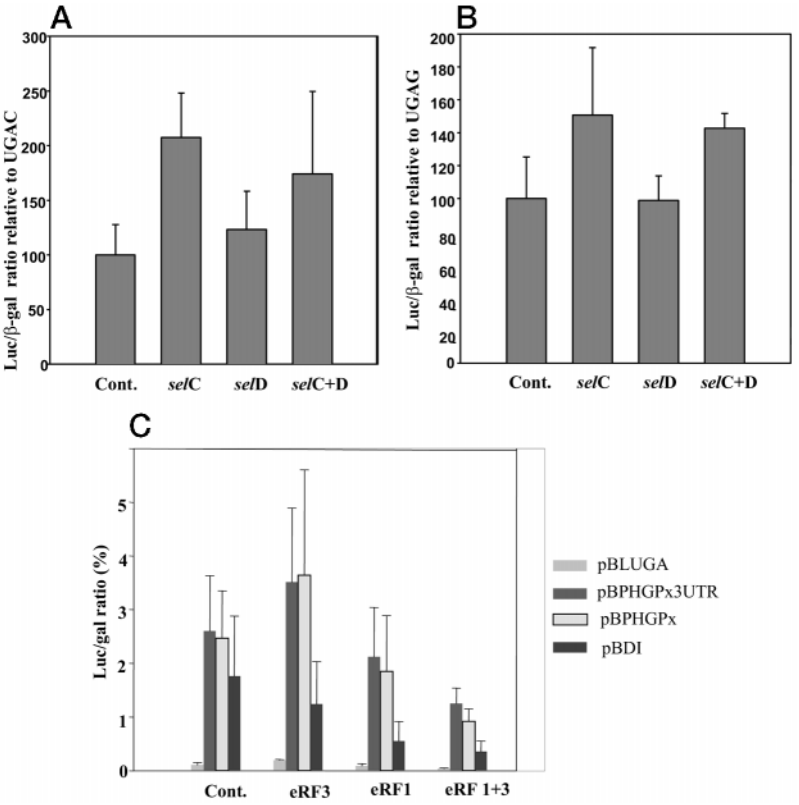


FIG. 4. **Co-transfection of *selC* and *selD* genes.** Transient co-transfections of BHK-21 cells were performed using the relevant plasmids (pH8B, pH8A, pH9A, pT12, and pT15) along with plasmids bearing *sel*C and *sel*D genes. Stop codon suppression efficiencies were estimated relative to the values obtained in control (*Cont.*) cells lacking co-transfected plasmids. Reporter constructs were used containing either UGAC (*A*) or UGAG (*B*) as selenocysteine incorporation site. Co-transfection experiments were performed using expression constructs encoding eRF1, eRF3, or both eRF1 and eRF3 (*C*). The *lacZ::luc* reporter constructs used had either no SECIS element in the 3′-UTR (pBPLUGA), only the PHGPx SECIS element (pBPPHGPx), the entire PHGPx 3′-UTR (pBPHGPx3U), or the rat 5′-deiodinase SECIS (pBDI).

structs were not reduced in response to the introduction of additional UGA codons (data not shown). Had there been general destabilization of the fusion mRNAs, we would have expected an associated loss of measurable β-galactosidase activity. Second, quantitative reverse transcriptase-PCR experiments also indicated that these changes were not attributable to variations in the abundance of the mRNAs encoded by the respective constructs (data not shown). Differences in the abundance of the encoded mRNAs were estimated to be no greater than 2-fold with respect to the non-UGA-carrying control.

One potential role of SECIS elements, at least theoretically, could be to suppress translation termination directly. However, the Western blotting data (for example see Fig. 5*C*) clearly showed that the yield of fusion protein was greatly reduced when multiple UGAs were present in the reporter mRNA. Given that expression through the *lacZ* domain was maintained (see above), this indicates that the primary non-SECIS-dependent event at the UGA codons was termination, as opposed to amino acid misincorporation.

DISCUSSION

We have investigated a range of parameters that could influence the efficiency of selenocysteine incorporation at single or multiple sites in selenoprotein genes. A number of factors are capable of attenuating the incorporation rate, but not sufficiently to suggest that the normal cellular level of selenocysteine incorporation is equivalent to 100% efficiency. Indeed, the data reported here, combined with the fact that there is considerable variation in the efficiencies supported by different SECIS elements (3, 24), indicate that termination occurs in parallel with selenocysteine incorporation at the UGA sites in selenoprotein mRNAs. There may be considerable variation in the relative rates of termination and selenocysteine incorporation at different UGA codons. It is striking that using the natural sequence contexts from selenoprotein genes in the expression constructs described here, we have obtained higher absolute levels of selenocysteine incorporation compared with the results obtained previously using short, self-designed UGA-containing sequences (24). However, the incorporation efficiencies obtained with transfected cells may not be representative of the maximum attainable values. Indeed, the incorporation efficiencies for natural genomic selenoprotein genes may be higher.

An alternative model for the mode of action of the SECIS element is that it not only promotes selenocysteine incorporation but also directly suppresses translation termination at UGA codons. However, unless there was a large increase in the mistranslation rate at the UGA site, any suppression on termination rate would have to feed back on the overall rate of protein synthesis. Because neither of these phenomena were observed (Fig. 5), we conclude that this model does not provide an adequate explanation of the results.

The competitive relationship between termination and selenocysteine incorporation can be modulated by a number of manipulations in the sequence region containing the UGA. Most strikingly, changes in the penultimate codons that precede the UGA can have a dramatic effect on the ability of the system to incorporate selenocysteine. However, we have found that termination-promoting codons, and thus the encoded amino acids, are evidently avoided among selenoprotein genes. The other *cis*-acting elements in the neighborhood of the UGA, the fourth nucleotide and downstream sequence and/or structure, exert a comparatively minor influence on selenocysteine incorporation. The observation that increased levels of eRFs have only a minimal observable effect on selenocysteine incorporation is not necessarily inconsistent with the existence of a competitive relationship with termination, because these factors may be close to effective saturation with respect to UGA-directed termination.

The consequences of the competition between termination and selenocysteine incorporation in terms of mRNA stability are difficult to predict. In principle, premature translational termination on an aberrant eukaryotic mRNA can trigger the

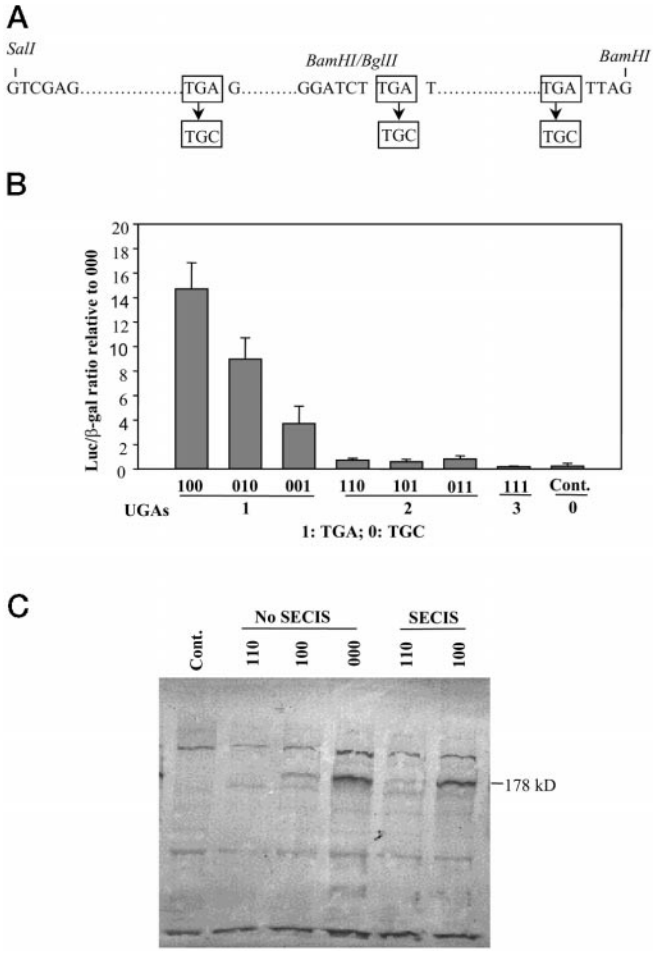


FIG. 5. **Effect of multiple UGAs on selenocysteine incorporation.** *A*, a segment of the *selP* gene (GenBank™ accession number Z11793; nucleotides 934–990), containing two UGA codons, was inserted into the *Bam*HI site of the reporter construct (Fig. 2B). This diagram indicates the relative positions of the PHGPx UGA codon and the two *selP* UGA codons. The synthetic copy of the selenoprotein P gene segment inserted was as follows: 5′-GA TCT TGA TGC TGC CAT TGT CGA CAT CTG ATA TTT GAA AAA ACA GGG TCT GCA ATC ACC TGA TTA G-3′. The selenocysteine incorporation sites are *underlined* or *boxed*. *B*, selenocysteine incorporation at multiple sites. Reporter plasmids pT18-pT32 (Table II) were used for transfection of BHK-21 cells, and SECIS-dependent stop codon suppression efficiencies were determined. TGA or TGC codons are indicated by 1 or 0 on the *x* axis. *C*, Western blotting indicates the relative amounts of β-gal::Luc fusion protein synthesized under the direction of constructs bearing one or two UGA codons. Extracts were prepared from transiently transfected BHK-21 cells, separated on a 6% SDS/polyarylamide gel, and proteins were blotted onto a polyvinylidene difluoride membrane. The β-gal-Luc fusion protein (approximately 178 kDa) was detected using anti-luciferase antibody. The control cells were not transfected. The presence of TGA or TGC at each position is indicated by a 1 or a 0, respectively. The SECIS-mediated incorporation accounts for a large proportion of the fusion protein synthesized in the presence of one or two UGAs.

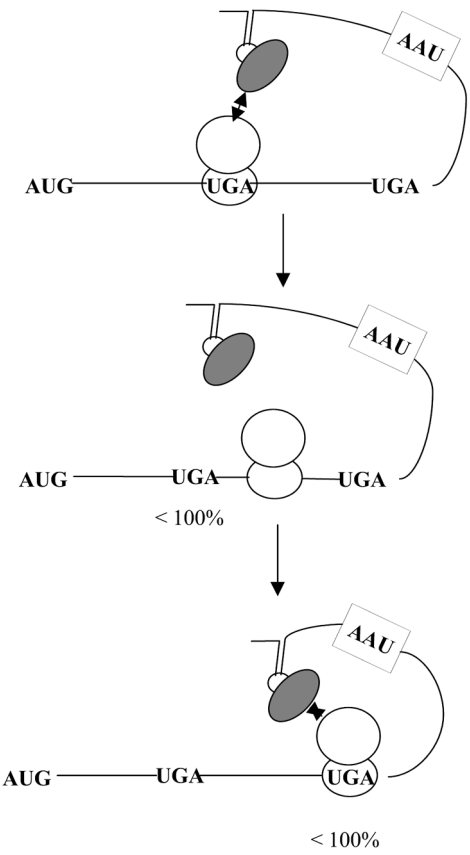


FIG. 6. **A nonprocessive model of selenocysteine incorporation.** Incorporation of selenocysteine at the first UGA in a multiple UGA mRNA is mediated by a SECIS-binding complex that associates with both the SECIS element and the elongating ribosome, whereby it is capable of suppressing UGA-dependent termination. Selenocysteine is only added substoichiometrically (efficiency, <100%) to the elongating polypeptide chain. For those chains where selenocysteine incorporation has been successful, elongation continues until the second UGA. The SECIS-bound complex may dissociate from the ribosome during this interim phase but then reassociate once the second UGA enters the ribosomal A site. The efficiency of incorporation at the second site is again <100%. This model predicts that the yield obtained with multiple UGAs will be a multiplicative function of the incorporation rates at the individual UGA sites. However, the operation of a mechanism that is partially processive under at least some conditions cannot be ruled out.

so-called nonsense-dependent mRNA decay pathway (37). However, selenoprotein mRNAs may constitute a special case, given that the internal stop codon fulfils a key function in the nonaberrant mRNA. So far, it has been found that the stability of glutathione peroxidase 1 mRNA decreases significantly under conditions of selenium limitation, whereas the destabilizing effect of ongoing termination under nonselenium-limited conditions seems to be small (38, 39). In contrast, even under selenium depletion conditions, neither PHGPx mRNA nor gastrointestinal glutathione peroxidase mRNA is destabilized by UGA-directed termination (38, 40). Our finding that the stability of the reporter mRNA used in this work is not markedly affected by changes in the efficiency or position of UGA-dependent termination is therefore consistent with the currently available data about the decay behavior of selenoprotein mRNAs.

We have established that termination and selenocysteine incorporation can occur at varying ratios at the same UGA. How does this affect the course of translation on selenoprotein mRNAs that have multiple internal UGAs? If substoichiometric incorporation occurs at a series of UGAs, the cumulative terminations at the successive sites would result in a significantly reduced yield of full-length selenoprotein. If the incorporation events are entirely independent, this yield would be equivalent to the multiplicative sum of the efficiencies at the individual UGAs. The loss of complete product could theoretically be limited if the incorporation process was processive, because this would mean that once a selenocysteine-incorporating ribosome complex had been established at the first UGA in the gene sequence, subsequent UGAs would all be recoded as selenocysteine.

However, our results are not consistent with the operation of a processive mechanism of selenocysteine incorporation. Even if the efficiencies seen here are not the maximum attainable, this can only have exacerbated (but not generated) the observed result that the combination of two or more UGAs gives

rise to additional suppression of the number of polypeptide chains that completes the open reading frame via (multiple) selenocysteine incorporation events. This phenomenon is therefore not attributable to a nonphysiological effect associated with partial saturation of the selenocysteine incorporation machinery and will apply to systems in which the absolute incorporation efficiency per site is greater than that estimated here. Processivity apparently does not provide the key to ensuring that multiple selenocysteine incorporation events occur successfully. The model that emerges from this envisages that termination can occur at any of the internal UGAs and that the interaction of the selenocysteine incorporation machinery with the translating ribosome at one UGA does not lead to enforced incorporation at other UGAs (Fig. 6). This may mean that there can be both assembly and disassembly of the selenocysteine incorporation apparatus on/from the ribosome each time a UGA is recoded. The major distinction to a processive mechanism is that the interaction of the selenocysteine incorporation components with the ribosome does not preprogram its subsequent behavior in a way that commits it to multiple selenocysteine incorporation events.

We have previously discussed the possibility that processivity might provide a mechanistic explanation for the generation of a selenoprotein such as SelP in adequate amounts (24). However, the current results indicate that the efficiency of selenocysteine incorporation at individual UGA sites is normally high enough to allow an adequate yield of complete SelP protein molecules in the absence of full processivity. At the same time, the proportion of prematurely terminated polypeptide chains generated from an mRNA bearing multiple UGA codons must be high. Given that genes such as SelP are likely to be rare, the resulting energetic wastage is presumably of little consequence in terms of the overall energetics of the cell.

Finally, suboptimal efficiencies of selenocysteine incorporation are not exclusive to eukaryotic systems, because the maximum efficiency of UGA decoding by SelB-GTP-Sec-tRNA^{Sec} achieved in *E. coli* so far is estimated to be 7% (41). It is tempting to rationalize such efficiencies purely in terms of the relative absolute abundance of the respective cellular components of the selenocysteine incorporation machineries in the two types of cell. However, as with other cellular systems, it is unlikely that a full understanding of rate control in the selenocysteine incorporation machinery can be achieved until more is known about the supramolecular organization of protein synthesis (41–43).

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REFERENCES

1. Böck, A., Forchhammer, K., Heider, J., Leinfelder, W., Sawers, G., Veprek, B., and Zinoni, F. (1991) *Mol. Microbiology* **5,** 515–520
2. Böck, A., Forchhammer, K., Heider, J., and Baron, C. (1991) *Trends Biochem. Sci.* **16,** 463–467
3. Low, C. C., and Berry, M. J. (1996) *Trends Bichem. Sci.* **21,** 203–208
4. Stadtman, T. C. (1996) *Annu. Rev. Biochem.* **65,** 83–100
5. Heider, J., Baron, C., and Böck, A. (1992) *EMBO J.* **11,** 3759–3766
6. Berry, M. J., Banu, L., Chen, Y., Mandel, S. J., Kieffer, J. D., Harney, J. W., and Larsen, P. R. (1991) *Nature* **353,** 273–276
7. Berry, M. J., Banu, L., Harney, J. W., and Larsen, PR. (1993) *EMBO J.* **12,** 3315–3322
8. Buettner, C., Harney, J. W., and Larsen, P. R. (1998) *J. Biol. Chem.* **273,** 33374–33378
9. Walczak, R., Westhof, E., Carbon, P., and Krol, A. (1996) *RNA* **2,** 367–379
10. Walczak, R., Carbon, P., and Krol, A. (1998) *RNA* **4,** 74–84
11. Zinoni, F., Heider, J., and Böck, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4660–4664
12. Baron, C., Heider, J., and Böck, A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4181–4185
13. Forchhammer, K., and Böck, A. (1991) *J. Biol. Chem.* **266,** 6324–6328
14. Leinfelder, W., Stadtman, T. C., and Böck, A. (1989) *J. Biol. Chem.* **264,** 9720–9723
15. Ehrenreich, A., Forchhammer, K., Tormay, P., Veprek, B., and Böck, A. (1992) *Eur. J. Biochem.* **206,** 767–773
16. Shen, Q., McQuilkin, P. A., and Newberger, P. E. (1995) *J. Biol. Chem.* **270,** 30448–30452
17. Shen, Q., Wu, R., Leonard, J. L., and Newberger, P. E. (1998) *J. Biol. Chem.* **273,** 5443–5446
18. Hubert, N., Walczak, R., Carbon, P., and Krol, A. (1996) *Nucleic Acids Res.* **24,** 464–469
19. Lesoon, A., Mehta, A., Singh, R., Chisolm, G., and Driscoll, D. M. (1997) *Mol. Cell. Biol.* **17,** 1977–1985
20. Copeland, P. R., and Driscoll, D. M. (1999) *J. Biol. Chem.* **274,** 25447–25454
21. Yamada, K., Mizutani, T., Ejiri, S., and Totsuka, T. (1994) *FEBS Lett.* **347,** 137–142
22. Berry, M. J., Maia, A. L., Kieffer, J. D., Harney, J. W., and Larsen, P. R. (1992) *Endocrinology* **131,** 1848–1852
23. Berry, M. J., Harney, J. W., Ohama, T., and Hatfield, D. L. (1994) *Nucleic Acids Res.* **22,** 3753–3759
24. Kollmus, H., Flohé, L., and McCarthy, J. E. G. (1996) *Nucleic Acids Res.* **24,** 1195–1201
25. Hill, K. E., Lloyd, R. S., Yang, J. G., Read, R., and Burk, R. F. (1991) *J. Biol. Chem.* **266,** 10050–10053
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 1.21–1.105, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Carbon, P., and Krol, A. (1991) *EMBO J.* **10,** 599–606
28. Low, S. C., Harney, J. W., and Berry, M. J. (1995) *J. Biol. Chem.* **270,** 21659–21664
29. Zhouravleva, G., Frolova, L., Le Goff, X., Le Guellec, R., Inge-Vechtomov, S., Kissilev, L., and Philippe, M (1995) *EMBO J.* **14,** 4065–4072
30. Tassan, J. P., Le Guellec, K., Kress, M., Faure, M., Camonis, J., Jacquet, M., and Philippe, M. (1993) *Mol. Cell. Biol.* **13,** 2815–2821
31. Laemmli, U. K. (1970) *Nature* **227,** 680–685
32. Towbin, H., Staehelin, T., and Gordon, G. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
33. Brigelius-Flohé, R., Aumann, K.-D., Blöcker, H., Gross, G., Kiess, M., Klöppel, K.-D., Maiorino, M., Rovri, A., Schuckelt, R., Ursini, F., Wingender, E., and Flohé, L. (1994) *J. Biol. Chem.* **269,** 7342–7348
34. McCaughan, K. K., Brown C. M., Dalphin, M. E., Berry, M. J., and Tate, W. P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5431–5435
35. Bjornsson, A., Mottagui-Tabar, S., and Isaksson, L. A. (1996) *EMBO J.* **15,** 1696–1704
36. Moustafa, M. E., El-Saadan, M. A., Kandeel, K. M., Mansur, D. E., Lee, B. J., Hatfield, D. L., and Diamond, A. M. (1998) *RNA* **4,** 1436–1443
37. Maquat, L. (1996) *Am. J. Hum. Genet.* **59,** 279–286
38. Bermano, G., Arthur, J. R., and Hesketh, J. E. (1996) *FEBS Lett.***387,** 157–160
39. Moriarty, P. M., Reddy, C. C., and Maquat, L. (1998) *Mol. Cell. Biol.* **18,** 2932–2939
40. Wingler, K., Böcher, M., Flohé, L., Kollmus, H., and Brigelius-Flohé, R. (1999) *Eur. J. Biochem.* **259,** 149–157
41. Suppmann, S., Persson, B. C., and Böck, A. (1999) *EMBO J.* **18,** 2284–2293
42. Negrutskii, B. S., Stapulionis, R., and Deutscher, M. P., (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 964–968
43. McCarthy, J. E. G. (1998) *Microbiol. Mol. Biol. Rev.* **62,** 1429–1553