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Dynamics and efficiency *in vivo* of UGA-directed selenocysteine insertion at the ribosome

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The kinetics and efficiency of decoding of the UGA of a bacterial selenoprotein mRNA with selenocysteine has been studied *in vivo*. A *gst–lacZ* fusion, with the *fdhF* SECIS element ligated between the two fusion partners, gave an efficiency of read-through of 4–5%; overproduction of the selenocysteine insertion machinery increased it to 7–10%. This low efficiency is caused by termination at the UGA and not by translational barriers at the SECIS. When the selenocysteine UGA codon was replaced by UCA, and tRNA^{Sec} with anticodon UGA was allowed to compete with seryl-tRNA^{Ser1} for this codon, selenocysteine was found in 7% of the protein produced. When a non-cognate SelB–tRNA^{Sec} complex competed with EF-Tu for a sense codon, no effects were seen, whereas a non-cognate SelB–tRNA^{Sec} competing with EF-Tu-mediated Su7-tRNA nonsense suppression of UGA interfered strongly with suppression. The induction kinetics of β-galactosidase synthesis from *fdhF′–′lacZ* gene fusions in the absence or presence of SelB and/or the SECIS element, showed that there was a translational pause in the fusion containing the SECIS when SelB was present. The results show that decoding of UGA is an inefficient process and that using the third dimension of the mRNA to accommodate an additional amino acid is accompanied by considerable quantitative and kinetic costs.

Keywords: EF-Tu/ribosomal pausing/SelB/selenocysteine/translation efficiency

Introduction

The biosynthesis and cotranslational incorporation of selenocysteine into *Escherichia coli* selenoproteins requires the products of four genes: *selA*, encoding the selenocysteine synthase which catalyses the formation of selenocysteine from serine attached to tRNA^{Sec}; *selB*, the selenocysteine-specific elongation factor; *selC*, the selenocysteine-specific tRNA^{Sec}; and *selD*, the selenophosphate synthetase which forms the selenium donor, selenophosphate, from ATP and reduced selenium (for a review see Baron and Bock, 1995). Recent analyses have shown that the elongation factor SelB has an N-terminal part structurally homologous to the three domains of elongation factor Tu (EF-Tu) (Hilgenfeld *et al*., 1996; Kromayer *et al*., 1996; Tormay *et al*., 1996) and a C-terminal extension involved in the binding of a mRNA secondary structure situated next to the selenocysteine UGA codon (Kromayer *et al*., 1996).

Genetic and biochemical studies suggest that SelB performs the same functions as EF-Tu. In its GTP-bound form, EF-Tu binds the aminoacylated tRNA forming the ternary complex and transfers the charged tRNA to the ribosome. In the initial selection step, non-cognate ternary complexes are dissociated from the ribosome. When cognate ternary complexes form proper anticodon–codon interactions, the GTPase activity of EF-Tu is stimulated, which induces a strong conformational change (Berchtold *et al.*, 1993). This leads to a reduction in the affinity for the bound tRNA, to the release of the tRNA to the ribosomal A site and the dissociation of EF-Tu–GDP from the ribosome. SelB differs from EF-Tu in several respects. It binds GTP with an ~10-fold higher affinity than GDP, and therefore does not require any guanine nucleotide release factor to displace GDP (Forchhammer *et al*., 1989). Additionally, SelB can only recognize the tRNA^{Sec} when it is charged with selenocysteine (Sec-tRNA^{Sec}) and not the precursor form charged with serine (Ser-tRNA^{Sec}; Forster *et al*., 1990), as opposed to EF-Tu which can recognize 40 different tRNA species charged with one of the standard amino acids. Most importantly, SelB does not form a ternary, but rather a quaternary complex with selenocysteyl-tRNA^{Sec}, GTP and the secondary structure of the mRNA (the SECIS element) immediately downstream of the UGA codon. This RNA structure directs selenocysteine insertion (Heider *et al*., 1992; Baron *et al*., 1993a; Ringquist *et al.*, 1994). The preformation of the quaternary complex requires stoichiometric amounts of the individual components and is essential for the decoding process (Tormay *et al*., 1996). A successful interaction with the ribosome, which is required for the stimulation of GTP hydrolysis (Huttenhofer and Bock, 1998), can occur only when the quaternary complex is formed.

There are, however, a number of open questions concerning the decoding process at the ribosome. It is not known whether the quaternary complex forms before the UGA reaches the ribosomal A site, and whether and when the mRNA structure is melted. Other unresolved questions concern the stimulus, the site and the time of GTP hydrolysis during the decoding process. To answer some of these open questions in this study we focus on the *in vivo* dynamics and efficiency of the decoding step catalysed by SelB.

A second important issue relates to the efficiency of selenocysteine insertion relative to that of the standard amino acids. It has been reported that read-through of the

UGA in mammalian selenoprotein mRNAs is only ~2% of that of a cysteine codon in the same position (Berry *et al.*, 1992; Kollmus *et al.*, 1996) and it is unknown whether this is due to chain termination at the UGA competing with selenocysteine insertion, or to some kinetic impediment of polypeptide chain initiation or elongation.

Results

Efficiency of selenocysteine insertion directed by SelB at the UGA codon

In order to determine the efficiency of selenocysteine insertion, we constructed a translation fusion between the glutathione *S*-transferase gene (*gst*) followed by the region of the *E.coli fdhF* gene necessary for selenocysteine insertion, and *lacZ* (Figure 1A). This construct allows the measurement of the amounts of both a protein terminated at the UGA codon and the read-through product resulting from selenocysteine insertion at the UGA.

Both products can be visualized and quantitated by Western blot analysis (Figure 1B) with α-Gst antibodies. The read-through product can also be quantified by measurement of β-galactosidase activity. The expression of the translation fusion is under control of the inducible *lac* promoter and protein production was determined 2 h after isopropyl-β-D-galactopyranoside (IPTG) induction.

In strain FM434 (wild type), the efficiency of selenocysteine insertion versus termination as measured by immunoblotting was only 4.8% (Table I). A similar value (3.9%) was found in strain JM109. Concomitant overproduction of SelB and $tRNA^{Sec}_{UGA}$ together with the selenocysteine synthase (SelA) gave 10.9%. The amount of full-length product as judged by β-galactosidase activity was in agreement with the data obtained in Western blot analysis.

The termination product can be seen as a double band corresponding to the initial termination product and a semi-stable degradation intermediate probably representing the Gst domain. In order to exclude the contribution of unspecific read-through of the UGA codon by suppressor tRNAs, expression was also measured in strain FM464 (Δ*selC*). In this strain, no full-length product could be detected.

To compare decoding efficiencies of SelB and EF-Tu and to ensure that the shorter product is indeed caused by termination at the UGA codon and not by ribosomal drop-off due to the mRNA structure or SelB binding, a construct was also used in which the selenocysteine UGA codon has been replaced by a UCA codon. No or very little drop off could be detected, and thus this cannot explain the low efficiency of selenocysteine insertion.

These results indicate that SelB-dependent decoding is very inefficient and that the inefficiency is correlated with termination at the UGA codon.

SelB-dependent decoding is less efficient than EF-Tu-dependent decoding

To determine the efficiency of decoding by the SelB quaternary complex compared with decoding by an EF-Tu ternary complex, a strain was constructed in which the chromosomal *selC* gene was mutated to express a $tRNA^{Sec}$, having the anticodon UGA and therefore being able to read the serine codon UCA (strain SM21, Figure 2C). This allows measurement of the competition between SelB and EF-Tu for the same codon. Plasmid pCCat carries a *fdhF′–′cat* fusion in which the UGA selenocysteine codon of *fdhF* has been replaced by UCA. When this plasmid is introduced into strain SM21, both Secys-$tRNA^{Sec}_{UGA}$ and Ser-$tRNA^{Ser1}$ can decode the UCA codon.

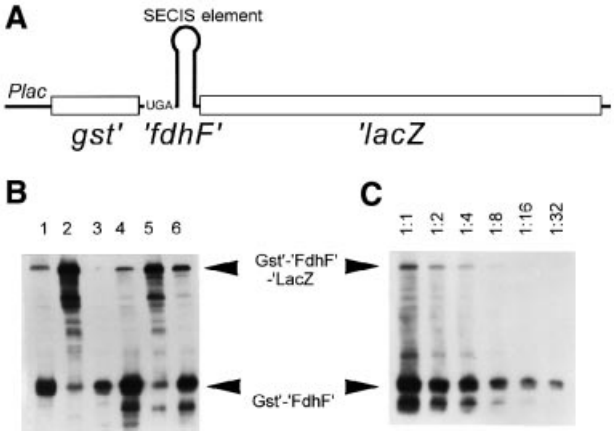

Fig. 1. (**A**) The Gst′–′FdhF′–′LacZ fusion of plasmid pSP03. (**B**) Immunoblot with α-Gst antibodies. Extracts were separated on SDS–11% polyacrylamide gels. (1) FM434/pSP03(UGA), (2) FM434/pSP04(UCA), (3) FM464(Δ*selC*)/pSP03, (4) JM109/pSP03, (5) JM109/pSP04, (6) JM109/pSP03/pSUABC. To avoid underestimation of the selenocysteine insertion efficiency, the degradation products were not included in the quantification, Table I, even though the Gst′–′FdhF′ protein is rather unstable. (**C**) To obtain an acurate quantification extracts were diluted in 2-fold steps, so that bands of equal intensities could be compared (the example shown is a dilution of an extract from FM434/pSP03).

To quantify the ratio of SelB-dependent selenocysteine insertion versus EF-Tu-dependent serine insertion, the amount of protein was measured as chloramphenicol acetyltransferase (Cat) activity and the fraction of the protein containing selenocysteine was determined by radioactive labelling with [^{75}Se]selenium (Table II; Figure 3). When protein is produced from an otherwise identical *fdhF′–′cat* fusion (on pWCat) with the wild-type UGA codon instead of UCA in strain MC4100, all protein giving Cat activity will contain selenocysteine. This protein was used as an internal standard in the measurements. The results are shown in Table II. In competition with EF-Tu, the UCA is decoded by SelB in 3% of the protein produced. One might argue that this difference is due to the fact that there are 80 times more EF-Tu molecules than SelB molecules in fast-growing *E.coli* cells (Forchhammer *et al.*, 1990). There are, however, only 6-fold more $tRNA^{Ser1}$ molecules than $tRNA^{Sec}$ molecules: 1296 and 219 molecules/cell, respectively (Dong *et al.*, 1996). In the presence of plasmid pWL107-2 SelB and the $tRNA^{Sec}_{UGA}$ are overproduced 40- and 50-fold, respectively, together with the selenocysteine synthase (SelA) (our unpublished results). Even under this condition the fraction of Fdh–Cat protein containing selenocysteine was only 7% (Table II). This suggests that the EF-Tu ternary complex is much more efficient than the SelB quaternary complex in decoding the UCA codon. Comparison of the Cat activities resulting from decoding of the *fdhF*(UGA)′–′*cat* or the *fdhF*(UCA)′–′*cat* mRNAs shows that selenocysteine insertion is an inefficient process also in the

Table I. Relative amounts of read-through product versus termination product upon translation of the *gst′–′fdhF′–′lacZ* fusion

Strain	Plasmid	Overproduction of SelA,B and C from pSUABC	Gst′–′FdhF′–′LacZ full length product[b] (%)
FM434 (wt)	pSP03 (UGA_{140})	−	4.8
FM434 (wt)	pSP04 (UCA_{140})	−	95.1
FM464 (*selC*)	pSP03 (UGA_{140})	−	n.d.[a]
JM109 (wt)	pSP03 (UGA_{140})	−	3.9
JM109 (wt)	pSP04 (UCA_{140})	−	96.0
JM109 (wt)	pSP03 (UGA_{140})	+	10.9

[a]Not detectable.
[b]100% is set as the sum of the Gst′–′FdhF′–′LacZ full-length protein and the Gst′–′FdhF′ termination product.

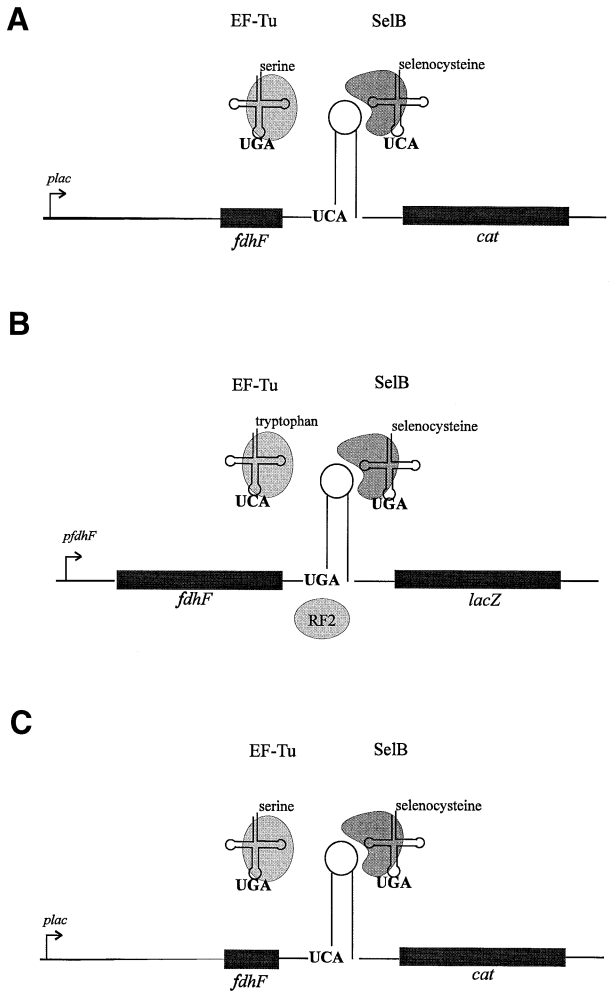

Fig. 2. Strategies for the *in vivo* measurement of competition between the two elongation factors EF-Tu and SelB. (**A**) Influence of the non-cognate SelB quaternary complex on UCA read-through by the cognate EF-Tu–GTP–Ser-tRNA^{Ser1} ternary complex. (**B**) Influence of the non-cognate SelB quaternary complex on suppression by the EF-Tu–GTP–Su7(op) ternary complex in competition with RF2. (**C**) Competition between a cognate SelB quaternary complex and the EF-Tu–GTP–Ser-tRNA^{Ser1} ternary complex. Cognate codon and anticodons are denoted in bold.

absence of EF-Tu competition, amounting to no more than 2.2% of the EF-Tu-tRNA^{Ser1} decoding efficiency. This supports the results obtained with the Gst–FdhF–LacZ fusions (Table I).

SelB does not lower the amount of EF-Tu-dependent decoding when the UGA selenocysteine codon is replaced by UCA

In an *in vitro* translation assay, even a 100-fold excess of a non-cognate tRNA fails to reduce the rate of protein synthesis due to high dissociation rates of non-cognate tRNAs from the ribosome in the initial selection process (Bilgin *et al*., 1988). Since the SelB–GTP–Sec-tRNA^{Sec} complex differs from EF-Tu ternary complexes in that it is anchored to the mRNA hairpin structure next to the selenocysteine UGA codon, it was of interest to see whether a non-cognate SelB quaternary complex could influence decoding by EF-Tu.

For an analysis, plasmid pCCat which carries the *fdhF*(TCA)′–′*cat* gene fusion was used. For the UCA codon in this fusion the wild-type tRNA$^{Sec}_{UCA}$ is non-cognate. The formation of the SelB quaternary complex at the mRNA hairpin will provide a high concentration of this non-cognate tRNA at the ribosomal A-site (Figure 2A) and thereby might prevent an efficient decoding by EF-Tu–Ser-tRNA^{Ser1}. In the presence of SelB (strain MC4100) the Cat activity expressed from the fusion is 10.4 μmol/min/mg, and in the absence of SelB (strain WL300) it is 10.1 μmol/min/mg, i.e. there is no difference in steady-state production of Cat activity dependent on the presence of SelB. Thus, the SelB quaternary complex does not lower the amount of read-through by EF-Tu–Ser-tRNA^{Ser}. This is in accordance with previous results (Tormay *et al*., 1996) which indicated that the non-cognate SelB complex is dissociated from the ribosome in a manner similar to that of a non-cognate EF-Tu ternary complex.

SelB interferes with EF-Tu-dependent suppression at the selenocysteine UGA codon

The measurement of the steady-state amount of FdhF–Cat protein, as demonstrated in the previous experiment, is a somewhat crude determination of whether the SelB quaternary complex can influence the decoding by EF-Tu. A reduction in the steady-state amount of Cat activity would be expected only if the SelB quaternary complex would cause peptide synthesis to be aborted by ribosomal drop-off, inhibit further translation initiation, or cause such long stalling of the ribosome that the downstream mRNA were to be degraded.

Therefore, a system was devised where termination through release factor RF2 would compete with EF-Tu decoding (Figure 2B). Plasmid pFM6047 carries an

Table II. Quantification of the efficiency of selenocysteine insertion

Strain	Plasmid encoded gene fusion	Over-production of SelA, SelB and $tRNA^{Sec}_{UGA}$	Cat activity (μmol/min/mg)[a]	Selenocysteine containing proportion of total protein[b] (%)
MC4100/pWCat	*fdhF*(TGA)′–′*cat*	−	0.2	100
SM21/pCCat	*fdhF*(TCA)′–′*cat*	−	9.0	3
SM21/pCCat, WL107-2	*fdhF*(TCA)′–′*cat*	+	5.5	7

[a]Cat activity and ^{75}Se incorporation (Figure 3) were measured in parallel cultures using the same inocula.
[b]Values were obtained from three independent labelling experiments, one of which is shown in Figure 3. 100% signifies that all the Cat produced contains selenocysteine.

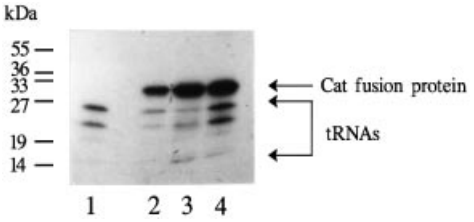

Fig. 3. Competition of EF-Tu and SelB. Autoradiograph of ^{75}Se-labelled FdhF–Cat fusion proteins separated on an SDS–15% polyacrylamide gel. Lane 1, WL300 (Δ*selB*)/pWCat (*fdhF*(TGA)′–′*cat*); lane 2, MC4100/pWCat; lane 3, SM21 ($selC_{TGA}$)/pCCat [*fdhF*(TCA)′–′*cat*]; lane 4, SM21 pCCat /pWL107-2 $selA,B,C_{TGA}$. tRNAs: tRNAs carrying the mnm^5Se^2U modification are also detected in this labelling experiment.

fdhF′–′lacZ fusion with the selenocysteine UGA codon and the mRNA hairpin structure directing selenocysteine insertion, and plasmid pFM6004 carries the same fusion with the hairpin structure deleted. This experiment was performed in strain SM21, which produces a chromosomally encoded $tRNA^{Sec}_{UGA}$ (with the anticodon altered to UGA), to avoid $tRNA^{Sec}$ recognizing the UGA codon and thereby adding to the amount of suppression. Here, the UGA codon can either be recognized by RF2, which leads to termination, or be decoded by the opal suppressor Su7-tRNA which leads to suppression of the UGA stop codon.

As expected, in the absence of the SECIS element there is no significant difference in UGA read-through, irrespective of whether SelB is present (Table III). However, for the fusion carrying the mRNA structure, the presence of SelB causes a 55% reduction in UGA suppression, probably by interfering with EF-Tu decoding and, hence, favouring termination by RF2.

In the absence of SelB, the read-through of the construct lacking the hairpin structure is almost 6-fold lower (with SelB 3.5-fold lower) than that of the construct with the hairpin. This might reflect the ability of the mRNA hairpin to interfere with RF2-dependent termination. Alternatively, the mRNA hairpin increases the stability of the message, as higher activity levels are seen in constructs with the mRNA structure also when the UGA codon has been mutated to UCA (Figure 5).

SelB causes ribosomal pausing

It is possible that SelB acts to decrease Su7 suppression at the UGA codon by interfering with EF-Tu binding to the ribosome, and thus stalling the ribosome at this codon. To measure such a stalling event, the rate of translation can be determined by measuring the induction time of β-galactosidase (Schleif *et al*., 1973). To see if SelB causes pausing of the ribosome, the induction kinetics of β-galactosidase (encoded by plasmid pSKS106) were compared with that of a hybrid β-galactosidase, in which 16 codons, containing the SelB binding hairpin structure and a UCA codon in place of the selenocysteine UGA codon, were inserted between the eighth and thirteenth codons of *lacZ* (encoded by plasmid pCT; Figure 4). In this fusion the wild-type $tRNA^{Sec}_{UCA}$ cannot decode the UCA codon but the SelB quaternary complex could interfere with EF-Tu decoding. Because of the 11 extra codons inserted, the induction time of the hybrid molecule could be expected to be 1–1.5 s longer than that of the native β-galactosidase (Bremer and Dennis, 1987). Such a small increase in induction time would, however, be difficult to detect in the β-galactosidase step time assay. The results obtained show that in the absence of SelB the induction time was practically identical for both molecules (Figure 5B; Table IV), but in the presence of SelB the appearance of β-galactosidase activity was delayed by ~8 s for the construct carrying the SelB mRNA binding site (Figure 5A). Thus, although the SelB quaternary complex cannot decode the UCA codon, it can delay the decoding by EF-Tu, leading to a dramatic translational pause.

The *lacZ* fusion construct of plasmid pWT is identical to that of plasmid pCT except for having the wild-type UGA codon, thus allowing wild-type $tRNA^{Sec}$ to read this codon. When the β-galactosidase step time of this construct was determined, it is found to be 12 s longer than that of wild-type β-galactosidase, which cannot exclusively be due to its slightly slower growth rate (Table IV). Therefore, a similar translational pause can be seen irrespective of whether EF-Tu–Ser-$tRNA^{Ser1}$ or SelB–Sec-$tRNA^{Sec}$ is reading the UCA/UGA codon. This pause is caused by SelB bound to the mRNA and not by the mRNA secondary structure itself, since it cannot be detected in the absence of the SelB protein.

The role of the mRNA secondary structure is more than just tethering of SelB: overproduction of SelB and selenocysteyl-$tRNA^{Sec}$ does not cause UGA suppression

It has been suggested that the function of the mRNA hairpin next to the selenocysteine UGA codon is to cause a local increase in the concentration of available SelB complexes at the UGA by tethering of SelB to the mRNA (Heider *et al*., 1992). If the role of the hairpin was only to increase the likelihood for a SelB complex to be available to the ribosome, it could be expected that a sufficiently high overproduction of SelB and selenocysteyl-$tRNA^{Sec}$ would lead to read-through of UGA

Table III. Influence of the non-cognate SelB quaternary complex on EF-Tu dependent UGA suppression

Strain	Relevant genotype	SelB binding mRNA motif	β-galactosidase activity (Miller units)
SM21/pSUsu7(op)/pFM6004	$SelC_{TGA}$, $selB^+$	no	146
SM22/pSUsu7(op)/pFM6004	$SelC_{TGA}$, *selB*	no	192
SM21/pSUsu7(op)/pFM6047	$SelC_{TGA}$, $selB^+$	yes	494
SM22/pSUsu7(op)/pFM6047	$SelC_{TGA}$, selB	yes	1110

Cells were grown to anaerobically to OD_{600} of maximally 0.15 to prevent loss of the plasmid pSUsu7(op).

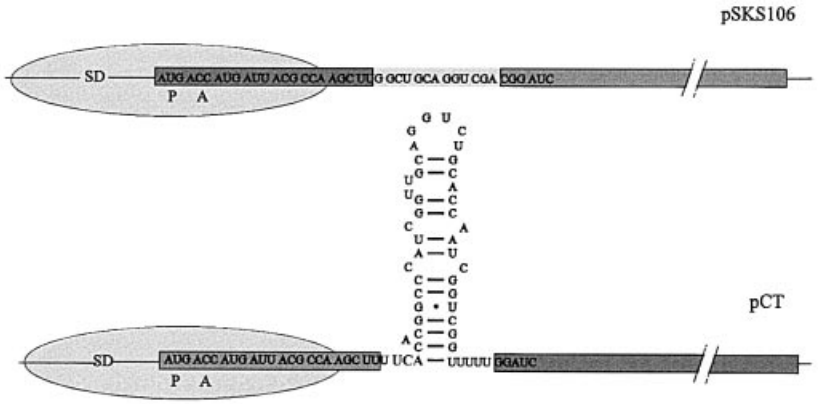

Fig. 4. Constructs used for determination of β-galactosidase induction times. The construction of plasmids is described under Materials and methods. The position of the *fdhF* hairpin structure relative to the initiating ribosome is indicated, assuming the ribosomal track to be located 16–19 bases downstream of the P-site codon (Hartz *et al*., 1989). The stem–loop is located six codons downstream from the A site and, thus, far enough not to interfere with the initiating ribosome.

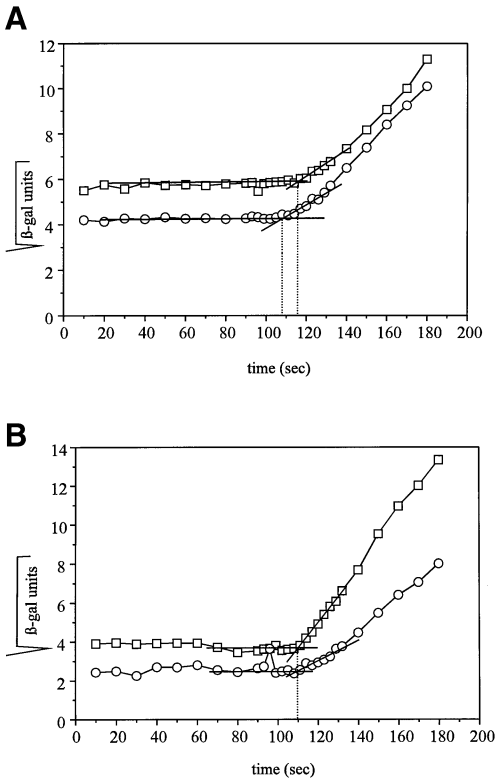

Fig. 5. Effect of the SelB–mRNA complex on the induction kinetics of β-galactosidase. Induction curves for strains FM434 wild-type (**A**) and SM81300 Δ*selB* (**B**). (○) pSKS106 (wild-type *lacZ*); (□) pCT (*fdhF′–′lacZ* gene cartridge). β-galactosidase units are according to Miller (1972).

codons in the absence of the hairpin structure. This suggestion was tested experimentally by overproducing wild-type $tRNA^{Sec}$ together with the wild-type SelB or truncated forms of SelB lacking the C-terminal mRNA binding domain. However, no SelB-dependent UGA codon suppression could be detected (Table V). This indicates that the function of the mRNA secondary structure is not solely to increase the concentration of SelB complexes at the ribosome, but that its binding induces a conformational switch to allow interaction between SelB and the ribosome (Klug *et al*., 1997; Huttenhofer and Bock, 1998).

Discussion

Co-translational incorporation of selenocysteine is inefficient

The results presented above have shown that selenocysteine insertion into polypeptides in *E.coli*, like in mammalia, is an inefficient process (Berry *et al*., 1992; Kollmus *et al*., 1996).

When the selenocysteine UGA codon together with the SECIS element was fused between *gst* and *lacZ* (Figure 1) an efficiency of only 3.9–4.8% (Table I) was measured. Upon overproduction of the components of the selenocysteine biosynthesis and incorporation machinery it can be raised to 10.9%. The low efficiency is caused by termination at the UGA codon and not by ribosomal drop-off due to the SECIS element, since when UGA is replaced by UCA, >95% full-length product is detected.

To define the basis of this inefficiency, we decided to use genetic systems that allow us to compare the dynamics of SelB-dependent selenocysteine incorporation relative to that of the elongation factor EF-Tu, and to study the kinetics of SelB-dependent decoding.

Table IV. β-galactosidase induction times

Strain	Relevant genotype	Generation time (min)	Induction time (s)[a]
FM434/pSKS106	*SelB*$^+$	20	108
FM434/pCT	*SelB*$^+$	20	116
SM81300/pSKS106	*SelB*	20	110
SM81300/pCT	*SelB*	20	110
FM434/pWT	*SelB*$^+$	24	120

[a]Values were obtained from measurements on at least three independent cultures. Values did not differ more than 1–2 s between experiments.

Table V. UGA suppression through SelB and tRNA^{Sec} overproduction

Strain	Plasmid	β-galactosidase activity (Miller units)
XAcB/ F′ B2 (*lacI*UCA189′–′*Z*)	–	230
XAcB/ F′ U4 (*lacI*UGA189′–′*Z*)	pSUsu7(op)	63
XAcB/ F′ U4 (*lacI*UGA189′–′*Z*)	pCB3 (*selC*) + pACYC184	1.3
XAcB/ F′ U4 (*lacI*UGA189′–′*Z*)	pCB3 + pWL143 (*selB*)	1.2
XAcB/ F′ U4 (*lacI*UGA189′–′*Z*)	pCB3 + pSelB588 (*selB′*)	1.5
XAcB/ F′ U4 (*lacI*UGA189′–‘*Z*)	pCB3 + pSelB474 (*selB′)*	1.4

The F′ episomes used are from Miller and Albertini (1983).

Competition between a cognate EF-Tu complex and a non-cognate SelB complex for a sense codon

It was shown previously that the overproduction of SelB did not lower the amount of decoding by EF-Tu of a UCA codon that replaced the UGA next to the SECIS element (Tormay *et al*., 1996). This study gives the same result, i.e. that a non-cognate SelB complex does not lower the amount of EF-Tu decoding of the UCA. Assuming that stimulation of the GTPase activity of SelB is required for a rapid dissociation of SelB from the mRNA, one would expect SelB to dissociate only slowly from the mRNA in the absence of a productive interaction of the SelB–GTP–Sec-tRNA^{Sec}–mRNA complex with the ribosome at a non-cognate UCA codon. It will, however, dissociate even in the absence of GTP hydrolysis and, therefore, no effect on EF-Tu decoding can be detected if the total amount of protein is quantified. In contrast, the kinetics of EF-Tu decoding are altered as a consequence of SelB competition (see below).

Competition between a cognate EF-Tu complex and a non-cognate SelB complex for a nonsense codon

When the codon for which EF-Tu and SelB are competing is a UGA codon (see Figure 2B), the presence of SelB leads to increased termination, showing that the non-cognate SelB quaternary complex can indeed interfere with EF-Tu decoding and reduce it to 45% (Table III). This again agrees well with earlier results of Tormay and Bock (1997). These authors have shown that suppression of the UGA codon of the *hydV* selenoprotein mRNA from *Desulfomicrobium baculatum* in *E.coli* is reduced to ~50% in the presence of the homologous SelB, but not in the presence of the heterologous *E.coli* SelB which does not bind to the SECIS element. It would seem that the SelB complex is slow in associating properly with the ribosome. Since in the absence of GTP hydrolysis the non-cognate SelB complex most likely remains associated with the SECIS element, this might lead to an inhibition of EF-Tu decoding until the SelB complex slowly dissociates from the mRNA. SelB interferes with EF-Tu-directed decoding during this time, but it does not interfere to the same extent with the action of RF2. Consequently, at the UGA codon the non-cognate SelB–GTP–Sec-tRNA$^{Sec}_{UGA}$–mRNA complex inhibits EF-Tu-dependent suppression and allows termination to occur (Table III).

Decoding in the presence of SelB is a slow process

Kinetic analysis of *fdhF–lacZ* induction shows that the ribosome is slowed down both in the wild-type situation, i.e. when the cognate SelB quaternary complex is decoding the UGA and when a EF-Tu ternary complex decoding UCA is ‘disturbed′ by a SelB bound to the mRNA secondary structure (Figure 5A). This is the first ribosomal pausing detected in connection with a RNA secondary structure *in vivo*, although such pausing has been shown to occur *in vitro* at frameshifting sites (reviewed by Farabaugh, 1996). The pausing at the selenocysteine insertion site is, however, not caused by the secondary structure itself, but by the SelB complex binding to it (Figure 5). It has been demonstrated that the selenocysteine UGA codon can be moved by one codon away in the 5′ direction from the SECIS secondary structure with only a 24–50% reduction in selenocysteine decoding (Heider *et al*., 1992; Chen *et al*., 1993). This suggests that the rather long ribosomal pause is caused by the ribosome stalling at two to three consecutive codons by the SelB quaternary complex interfering with EF-Tu decoding, and might explain why a pause is still seen when the UGA codon is decoded by SelB rather than by EF-Tu (Table IV).

Direct competition between EF-Tu and SelB for the same codon

Considering the dramatic effect SelB exerted on EF-Tu decoding it was rather surprising to find that when SelB and EF-Tu compete for the same sense codon (see Figure 2C), with SelB–GTP–Sec-tRNA$^{Sec}_{UGA}$ inserting selenocysteine and EF-Tu–GTP–Ser-tRNA^{Ser1} inserting serine, only 3% of the protein formed contained selenocysteine.

When SelB, the $tRNA^{Sec}$ and the selenocysteine synthase are overproduced >40-fold, the fraction of selenocysteine insertion only increased to 7%, suggesting that in this case the inefficiency of selenocysteine insertion is not due to limiting amounts of the components of selenium metabolism (Table II). These values agree well with those obtained with the GST–LacZ fusions, although in that case the efficiencies were determined in the absence of EF-Tu competition. The comparison of decoding by EF-Tu–GTP–Ser-$tRNA^{Ser1}$ at UCA or by SelB–GTP–Sec-$tRNA^{Sec}$ at UGA, shows the efficiency of the SelB complex to be only ~2% of that of EF-Tu. This closely resembles the mammalian selenocysteine incorporating system, which displays efficiencies of 1–3% (Berry *et al*., 1992; Kollmus *et al*., 1996). In the mammalian system, the basis for this low efficiency is thought to reside in limiting amounts of the components involved in selenium metabolism and in the high efficiency of translation termination (Berry *et al*., 1992; Kollmus *et al*., 1996), although the amount of mammalian $tRNA^{Sec}$ seems not to be limited (Moustafa *et al*., 1998). The experiments presented here indicate that in *E.coli*, it is the decoding by the SelB quaternary complex that is the limiting factor for selenocysteine insertion rather than the cellular concentration of individual components of the complex.

These data also promote some speculation on the decoding activity of SelB. The low efficiency could rest on the fact that the SelB complex is slow in finding its proper position at the ribosome. It is also possible that the $tRNA^{Sec}$, the structure of which differs significantly from that of other tRNAs (Baron *et al*., 1993b), is slower in binding properly to the ribosomal A site. Another explanation would be based on a postulated channeling process at the ribosome. Deutscher and co-workers (Negrutskii *et al*., 1995; Stapulionis and Deutscher, 1995) have suggested that in eukaryotic cells there is a loose multiprotein complex around the ribosome channeling tRNAs from tRNA synthetases to the ribosome and back again, with EF-1α as the carrier molecule. Also, Spirin and co-workers have shown in continuous flow cell-free systems that tRNAs, initiation factors and elongation factors in eukaryotes and in *E.coli* do not exist as freely diffusible individual molecules, but are tightly bound to the protein synthesis machinery (Baranov and Spirin, 1993). If such a dynamic multi-protein translation complex exists in *E.coli*, it might be that SelB as it sits on the mRNA is impeded from entering this multi-protein complex where ternary complexes are channeled to the ribosome, but still is able to interfere with EF-Tu ternary complexes within the channel.

The function of the SECIS element

The present work also supports previous studies and presents new information on the function of the SelB×SECIS interaction. During strong overproduction of SelB and its tRNA, there is no selenocysteine insertion at the UGA codon if the mRNA binding motif is absent (Table V), indicating that the mRNA motif is not merely increasing the concentration of SelB quaternary complex in the proximity of the UGA codon. SelB has to bind to the mRNA to be able to interact with the ribosome, and it must be assumed that it undergoes a conformational change upon binding the mRNA (Klug *et al*., 1997; Huttenhofer and Bock, 1998). In accordance with this, the binding of the mRNA motif strongly enhances the ribosome-stimulated GTPase activity of SelB *in vitro* (Huttenhofer and Bock, 1998). Thus, the mRNA structure also constitutes a security switch to avoid reading of ordinary UGA stop codons as selenocysteine.

Conclusions

The co-translational incorporation of selenocysteine is an inefficient process. Irrespective of whether an EF-Tu ternary complex is allowed to compete with the SelB quatenary complex, the efficiency is ~2–5%. Even when SelB, $tRNA^{Sec}$ and the selenocysteine synthase is over-produced ~40- to 50-fold, the efficiency of selenocysteine incorporation remains as low as 7–10%. This inefficiency is paired with a slow rate of the insertion process, caused by the fact that the ribosome stalls for a considerable time at the UGA codon.

At present we can only speculate on the step(s) which kinetically limits the incorporation process. Each of the following partial reactions, either singly or in combination, could be involved: docking of the SelB quaternary complex in a correct position to the ribosome; or induction of the conformational switch of SelB which stimulates GTPase activity and causes release of the sec-$tRNA^{Sec}$ dissociation of SelB from the mRNA. Further work is required to differentiate between these possibilities. Overall, however, one can state that the accommodation of an additional amino acid in the genetic decoding by using three-dimensional information in the mRNA is accompanied by considerable efficiency and kinetic costs compared with the linear readout of triplets.

Materials and methods

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this work are listed in Table VI. Bacteria were grown aerobically in TP medium (1% tryptone, 0.1 M potassium phosphate pH 6.5, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 μm Na_2SeO_3, 0.4 μM H_3BO_3, 30 nM $CoCl_2$, 10 nM $CuSO_4$, 10 nm $ZnSO_4$, 80 nm $MnCl_2$, 10 μM $FeCl_3$, 0.5% glycerol) (Heider *et al*., 1992) or Luria–Bertani (LB) medium (Bertani, 1951) and anaerobically in TGYEP medium (1% tryptone, 0.5% yeast extract, 0.4% glucose. 0.1 M potassium phosphate pH 6.5, 1 μM Na_2 SeO_3) (Begg *et al*., 1977) supplemented with 30 mM formate. Antibiotics kanamycin, ampicillin and chloramphenicol were added to concentrations of 50, 100 and 30 μg/ml, respectively.

Construction of strains SM21, SM22 and SM81300

To generate a strain harbouring a chromosomal copy of $tRNA^{Sec}_{UGA}$, the *Eco*RI–*Sma*I *selC* fragment of plasmid pMN60 was substituted by the corresponding fragment containing the TGA mutation from plasmid $pSelC_{TGA}$. The 1.3 kb *selC Hin*dIII–*Sph*I fragment of the resulting construct was then transferred to pMAK705 and integrated into the chromosome of *E.coli* MC4100 by the gene replacement method of Hamilton *et al.* (1989). The loss of wild-type *selC* in the resulting strain SM21 was seen as loss of FDH_H activity whereas integration of $selC_{TGA}$ was detected by its ability to confer FDH_H activity in the presence of plasmid pBFM203. This plasmid encodes the *fdhF* mutant TCA_{140} and needs the complementary $tRNA^{Sec}_{UGA}$ derivative for selenocysteine insertion at this position. The corresponding Δ*selB300:kan* strain SM22 was constructed by transduction of SM21 with a P1 lysate of WL300 by the method of Miller (1972).

Strain SM81300 was constructed by transferring the episome F′: $lacI^q$,Cm^R from strain FM434 into strain WL81300.

Construction of plasmids pCCat, pWCat, pCT, pSUsu7 and pWL107-2

For construction of the *fdhF′–′cat* gene fusion on pWCat, a 126 bp *Hin*dIII–*Bam*HI fragment from pFM320 carrying the stem–loop structure

Table VI. Strains and plasmids

Strain or plasmid	Genotype or phenotype	Reference or source
E.coli K12 strains		
FM434	(Δ*argF–lac*)U169, *araD139, flbB5301, ptsF25, deoC1, relA1, rpsE13,* (Δ*srl*–recA)306::Tn*10*, F′: *lacI*^{q}, Cm^R	Zinoni *et al*. (1990)
FM464	(Δ*argF–lac*)U169, *araD139, flbB5301, ptsF25, deoC1, relA1, rpsE13,* (Δ*srl–recA)306::*Tn*10*, Δ*selC::kan*, F′: *lacI*^{q}, Cm^R	Zinoni *et al*. (1990)
JM109	*recA1, endA1, gyrA96, thi-1, hsdR17, relA1, supE44,* Δ(*lac–proAB*); F′ *traD36, proAB+, lacIq, lacZΔM15*	Yanisch-Perron *et al.* (1985)
MC4100	(Δ*argF–lac*)U169, *araD139, flbB5301, ptsF25, deoC1, relA1, rpsL150, thi*(*?*)	Casadaban and Cohen (1979)
SM21	MC4100*selC*$_{TGA}$	this study
SM22	SM21 Δ*selB300::kan*	this study
SM81300	Δ*selB300::kan, araD139*, (Δ*argF*–lac)U169, *ptsF25, relA1, deoC1, flbB5301, rpsE13,* (Δ*srl–recA)306::*Tn*10*, F′: *lacI*^{q}, Cm^R	this study
WL300	Δ*selB300::kan*, (Δ*argF–lac*)U169, *araD139, flbB5301, ptsF25, deoC1, relA1, rpsL150, thi*(*?*)	Leinfelder *et al.* (1988)
WL81300	Δ*selB300::kan* (Δ*argF–lac*)U169, *araD139, flbB5301, ptsF25, deoC1, relA1, rpsE13,* (Δ*srl–recA)306::*Tn*10*,	Tormay and Bock (1997)
XAc	(Δ*lac–pro*)X111, *ara, thi, argE*(am), *gyrA, rpoB*	Coulondre and Miller (1977)
XAcB	Δ*selB300::kan*, (Δ*lac–pro*)X111, *ara, thi, argE*(*am*), *gyrA, rpoB*	this study
Plasmids		
pACYC184	Cm^R, Tc^R	Chang and Cohen (1978)
pBFM203	Ap^R, *fdhF*(TCA140)	Baron *et al*. (1990)
pBR322	Ap^R, CmR	Bolivar *et al*. (1977)
pCB3	Ap^R, *selC*	Baron *et al*. (1990)
pCCat	Ap^R, *fdhF*(TCA)′–′*cat* with the selenocysteine TGA$_{140}$ codon mutated to TCA	this study
pCT	as pWT but with the selenocysteine TGA140 codon mutated to TCA	this study
pFM320	Ap^R, (*lacZ–fdhF*)117*lacYA*	Zinoni *et al*. (1990)
pFM6004	Ap^R, *fdhF′–′lac* fusion 4 nucleotides downstream of the TGA in *fdhF*	Zinoni *et al*. (1990)
pFM6047	Ap^R, *fdhF′–′lac* fusion 47 nt downstream of the TGA in *fdhF*, including the SECIS element	Zinoni *et al*. (1990)
pMAK705	Cm^R, *lac′IPOZ′*α	Hamilton *et al*. (1989)
pSelB474	Cm^R, *selB′* truncated after codon 474	Tormay *et al*. (1996)
pSelB588	Cm^R, *selB′* truncated after codon 588	Tormay *et al*. (1996)
pSelC$_{TGA}$	Ap^R, *selC*$_{TGA}$	Baron *et al*. (1990)
pSKAGS	Ap^R, P*lac–gst′–′lacZ* fusion, pBR322 derivative	Wills *et al*. (1997)
pSKS106	Ap^R, *lacZYA*	Shapira *et al*. (1983)
pSP03	Ap^R, *gst′–′fdhF′–′lacZ* translation fusion	this study
pSP04	Ap^R, *gst′–′fdhF*(UCA)′–′*lacZ* translation fusion	this study
pSU2719	Cm^R, *lac′IPOZ′*α	Martinez *et al*. (1988)
pSUsu7(op)	Cm^R, su7-TGA	this study
pSWC101	Tet^R, su7-TGA	Raftery *et al*. (1984)
pUC19	Ap^R, *lac′IPOZ′*α	Yanisch-Perron *et al.* (1985)
pWCat	Ap^R, *fdhF*(TGA)′–′*cat*	this study
pWL107	Cm^R, pACYC184 derivative, *selAB*	Leinfelder *et al*. (1988)
pWL107-2	Tet^R, *selAB*, *selC*$_{TGA}$	this study
pWL143	Cm^R, *selB*	Tormay and Bock (1997)
pWT	Ap^R, *fdhF*(TGA)′–′*cat*	Heider *et al*. (1992)

necessary for selenocysteine insertion was ligated with plasmid pUC19 that had been cut with the same enzymes. The *cat* gene was amplified from plasmid pACYC184 by PCR (Innis *et al*., 1990) using the synthetic oligonucleotides SM15 and SM16, thereby deleting the ATG start codon of the *cat* gene. The PCR fragment was subsequently restricted with *Bam*HI and *Stu*I and ligated with the above described *fdhF* stem–loop carrying pUC19 construct, which had been cut with *Bam*HI–*Sma*I. The resulting plasmid pWCat containing the *fdhF′–′cat* gene cartridge was subjected to PCR mutagenesis in order to replace the UGA$_{140}$ selenocysteine codon by a UCA serine codon. The PCR fragment obtained with the oligonucleotides SM13 and SM16 (Table VII) was restricted with *Hin*dIII–*Stu*I and ligated with *Hin*dIII–*Sma*I cut plasmid pUC19, resulting in plasmid pCCat. For determination of polypeptide chain growth rate, the *fdhF′*(TCA$_{140}$)–′*lacZ* gene cartridge on plasmid pCT was constructed by substituting the UGA$_{140}$ codon in plasmid pWT by M13 mutagenesis (Messing, 1983) and ligating the appropriate stem–loop containing 48 bp *Hin*dIII–*Bam*HI fragment with plasmid pSKS106 treated with the same enzymes.

To measure UGA codon suppression, the 0.3 kb *Eco*RI fragment harbouring opal suppressor tRNA su7(op) from plasmid pSWC101 was filled in with Klenow fragment of DNA polymerase I and ligated with *Sma*I cut plasmid pSU2719.

To overproduce selenocystein synthase, SelB and tRNA$^{Sec}_{UGA}$ from one plasmid, the *Bam*HI fragment from plasmid pSelC$_{TGA}$ carrying the *selC*$_{TGA}$ gene was transferred to plasmid pWL107 harbouring *selAB*. As this pACYC184 derivative carries a *cat* selection marker which would interfere with the *fdhF′–′cat* gene expression experiments, the *cat* gene was destroyed by *Eco*RI cleavage. After filling in the 5′-overhanging *Eco*RI ends by the Klenow fragment of DNA polymerase I, a *Ssp*I–*Pvu*II fragment from pBR322 with a tetracyclin resistance gene was inserted at this site, giving plasmid pWL107-2.

Construction of plasmids pSP03 and pSP04

Plasmids pSP01 [*hycA–fdhF*(UGA)–*cat*] and pSP02 [*hycA–fdhF*(UCA)–*cat*] are both pUC19 derivatives. A 477 bp fragment of the *E.coli hycA* gene was amplified by PCR using the primers SMA1 and SMA2 (Table VII). This PCR fragment was cleaved with *Hin*dIII and ligated into the *Hin*dIII site of pWCat, to form plasmid pSP01. Plasmid pSP01 was then cut with *Bsg*I and *Bsm*FI, which cleave on opposite sides of the selenocysteine codon. This fragment was replaced by inserting the oligonucleotides FDHF and FDHB (Table VII), which form a double-strand fragment identical to the one excised, except for the selenocysteine UGA codon being altered to UCA. The *fdhF* fragments thus inserted between *hycA* and *cat* in plasmids pSP01 and pSP02 were amplified by PCR using primers HYC and CAT and inserted between the *gst* and *lacZ* genes of pSKAGS (Wills *et al*., 1997) to form plasmids pSP03 and

Table VII. Oligodeoxyribonucleotides used

Name	Sequence
SM13	GCGCAAGCTTCTACGTAATGCAAAAATTTGCGCGCGCCGTTATTGGTACCCAATAACGTTGACCTGCTGCGCTCGTGTCTCCACACGG
SM15	GCGCGGATCCCGGAGAAAAAAAATCACTG
SM16	GCGCAGGCCTTACGCCCCGCCCTGCCA
SMA1	TTTAAGCTTGCTGCCGGGCTTTGTCCCTTT
SMA2	TTTAAGCTTAATGACTATTTGGGAAATAAG
FDHF	CCGTGTGAGACACGAGCGCAGCAGTCAACGTTATTGGTACCAATAACGGCGCGCGCAAATTTTTGCATTACATAGAAGCT
FDHB	AGCAAGCTTCTATGTAATGCAAAAATTTGCGCGCGCCGTTATTGGTACCAATAACGTTGACTGCTGCGCTCGTGTCTCACACGGCC
CAT	CAAAGCCCGGCAGCAAGC
HYC	CCGGAATCCGCGGGGCCCTTACCGACC

pSP04. Sequencing confirmed the presence of the in-frame UGA or UCA codon.

Chloramphenicol acetyltransferase assay

Chloramphenicol acetyltransferase activity was measured by the method of Shaw (1975). This spectrophotometric assay is based on the reaction of free sulfhydryl groups of CoA, generated by the acetyltransferase reaction and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The concomitant generation of free 5-thio-2-nitrobenzoate is measured as increase in extinction at 412 nm. Assays were performed in 100 mM Tris–HCl pH 7.8, 100 µM acetyl-CoA, 0.4 mg/ml DTNB and 1–20 µl freshly prepared S30 extracts of cells, started by addition of 5 mM chloramphenicol and followed in a Beckman spectrophotometer (DU 640) at room temperature.

β-galactosidase activity assay

β-galactosidase activity was determined as described by Miller (1972). The cells were lysed by the method of Putnam and Koch (1975).

In vivo labelling with [^{75}Se]selenite

Selenium labelling was performed as described by Cox *et al*. (1981). Cells were grown aerobically for 8 h in TP medium (Heider *et al*., 1992) supplemented with 0.4% glycerol and 50 µg/ml cysteine. [^{75}Se]selenite was added at 1 µM and a specific activity of 1.000 µCi/µmol. Cells were harvested and resuspended in 150 µl sample buffer per ml and per optical density of 1 to adjust the protein concentration of the different samples. Equal amounts were then subjected to SDS–PAGE and radioactively labelled proteins were visualized by autoradiography and quantified using a molecular dynamics densitometer.

Determination of polypeptide chain growth rate

Determination of the polypeptide chain growth rate of β-galactosidase was essentially as described by Schleif *et al*. (1973) using the lysis procedure of Putnam and Koch (1975). Cells were grown at 37°C to an OD_{60} of 0.25. Then cAMP was added to a concentration of 1 mM. After 20 min β-galactosidase production was induced by the addition of IPTG to 0.5 mM. Samples were taken every 10 s, and after 90 s every 3 s, and immediately cooled on ice in lysis buffer. The induction time of each strain was measured at least three times.

Quantification of SelB and tRNA^{Sec}

To quantify the amounts of selenocysteine-specific tRNA and elongation factor in the cell during overproduction from plasmid pWL107-2, the following procedures were employed.

Quantification of the elongation factor SelB: dilution series of S30 protein extracts from the strains of interest were separated by SDS–PAGE. The proteins were blotted onto a nitrocellulose filter (Schleicher and Schuell), and Western immunoblot was performed with SelB polyclonal antibodies. Bound antibody was detected with the ECL kit (Amersham Buchler, Braunschweig). Known amounts of purified amounts of *E.coli* SelB served as internal standard (not shown).

Quantification of the tRNA^{Sec}: total RNA was extracted from 0.3 ml of cells grown to an OD_{600} of 2.0 by a modified method of Varshney *et al.* (1991), using TE buffer (10 mM Tris, 1 mM EDTA pH 8) minstead of acidic buffer. Total tRNA was resuspended in 20 µl TE buffer. Samples (1 and 10 µl) were separated on a 12% polyacrylamide–7% urea gel and blotted onto a Nytran filter (Schleicher and Schuell). The filter was probed with the oligonucleotide 5′-ACAGGAGTCG-AACCTGC-3′ complementary to the TψC stem and loop, of tRNA^{Sec}. The oligonucleotide was 5′-end labelled with [γ-^{32}P]ATP (NEN, Dupont) and polynucleotide kinase (Biolabs). The hybridization to purified tRNA^{Sec} was used as reference (not shown).

Quantification of Gst fusion proteins

To quantify the products from the *gst′–′fdhF′–′lacZ* fusion vectors, cells were grown at 37°C to an OD_{600} of 0.3 and induced with 0.5 or 0.05 mM IPTG. Samples were taken for β-galactosidase activity measurement and for immunoblotting both before induction and when the cells were harvested 2 h after induction. For immunoblotting, cells were boiled in SDS sample buffer and samples were separated on SDS–11% polyacrylamide gels (Laemmli and Favre, 1973).

Proteins were blotted onto a Hybond-P filter (Amersham) using a Trans-Blot Cell (Bio-Rad) using a buffer containing 39 mM glycine, 48 mM Tris base, 0.05% SDS, 20% methanol. After blotting filters were dried, re-wet and stained with amido black. All antibody interaction were done in TBS-T buffer (20 mM Tris–HCl pH 7.5, 137 mM NaCl, 1% Tween 20). The primary antibodies used for detection of the Gst fusion products were anti-Gst antibodies developed in rabbit (Sigma). Secondary antibodies and luminol detection system were from the BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim) and used according to the manufacturer. To eliminate inaccuracies caused by unproportionally low binding of antibodies to heavy protein bands, samples were diluted so that bands of similar intensity could be analysed when determining the ratio between the terminated Gst′–′FdhF′ and full length Gst′–′FdhF′–′LacZ products (Figure 1C).

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