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Coding from a distance: dissection of the mRNA determinants required for the incorporation of selenocysteine into protein

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Incorporation of selenocysteine into proteins is directed by specifically 'programmed' UGA codons. The determinants for recognition of the selenocysteine codon have been investigated by analysing the effect of mutations in *fdhF*, the gene for formate dehydrogenase H of *Escherichia coli*, on selenocysteine incorporation. It was found that selenocysteine was also encoded when the UGA codon was replaced by UAA and UAG, provided a proper codon-anticodon interaction was possible with $tRNA^{Sec}$. This indicates that none of the three termination codons can function as efficient translational stop signals in that particular mRNA position. The discrimination of the selenocysteine 'sense' codon from a regular stop codon has previously been shown to be dependent on an RNA secondary structure immediately 3′ of the UGA codon in the *fdhF* mRNA. It is demonstrated here that the correct folding of this structure as well as the existence of primary sequence elements located within the loop portion at an appropriate distance to the UGA codon are absolutely required. A recognition sequence can be defined which mediates specific translation of a particular codon inside an mRNA with selenocysteine and a model is proposed in which translation factor SELB interacts with this recognition sequence, thus forming a quaternary complex at the mRNA together with GTP and selenocysteyl-$tRNA^{Sec}$.

Key words: mRNA context/RNA binding protein/RNA secondary structure/termination codon/translational control

Introduction

Escherichia coli is able to synthesize three selenocysteine containing polypeptides which are constituent subunits of the formate dehydrogenase (FDH) isoenzymes, FDH_H, FDH_N (Cox *et al.*, 1981) and FDH_O (Sawers *et al.*, 1991). The biosynthesis of selenocysteine and its incorporation into protein is accomplished by the products of the *sel* genes (Böck *et al.*, 1991). During biosynthesis an ester-bonded seryl residue at a special tRNA ($tRNA^{Sec}$) is converted to a selenocysteyl residue by selenocysteine synthase, the *selA* gene product. This reaction proceeds through an aminoacrylyl-tRNA intermediate (Forchhammer and Böck, 1991) and involves a phosphate-activated selenium donor molecule which is synthesized by the *selD* gene product (Forchhammer *et al.*, 1991; Ehrenreich *et al.*, 1992; Veres *et al.*, 1992).

The resulting selenocysteyl-$tRNA^{Sec}$ is specifically recognized by an alternate elongation factor, SELB, which has been shown to replace EF-Tu in binding this particular tRNA and delivering it to the ribosome (Forchammer *et al.*, 1989; Förster *et al.*, 1990).

Specific incorporation of selenocysteine into distinct proteins is directed by in-frame UGA codons in the mRNAs derived from all known selenoprotein genes (Böck *et al.*, 1991). Since UGA codons are normally used as translation termination codons, the existence of a specific mechanism to discriminate them from UGA codons directing selenocysteine insertion is obvious (Böck *et al.*, 1991).

This discrimination does not require a UGA codon *per se*, because selenocysteine incorporation into the *E.coli fdhF* gene product still occurred when the UGA was mutated. After mutation at the third ('wobble') position (to a cysteine codon, UGC or UGU), significant selenocysteine incorporation could be observed in wild-type strains; this is reduced, but not abolished by restrictive *rpsL* alleles (Zinoni *et al.*, 1987, 1990). After mutation at the second position to a serine (UCA) codon, selenocysteine incorporation was abolished but could be rescued when a compensatory exchange was introduced into the anticodon of $tRNA^{Sec}$ (Baron *et al.*, 1990). In these cases selenocysteine insertion into polypeptides by $tRNA^{Sec}$ efficiently competed with the insertion of cysteine or serine via the 'normal' tRNAs (Baron *et al.*, 1990). These data suggest that additional mRNA elements outside of the UGA codon are responsible for the recognition of selenocysteine codons. Indeed, it was demonstrated recently for the *E.coli fdhF* gene (encoding the 80 kDa subunit of FDH_H) and the *fdnG* gene (Berg *et al.*, 1991b) that the presence of a 3′-flanking sequence is required in addition to the UGA codon to obtain efficient incorporation. This mRNA region correlates with the existence of a putative mRNA secondary structure (Zinoni *et al.*, 1990; Berg *et al.*, 1991b).

Independent support for the relevance of this structure came from the generation of a new selenoprotein by mutating the gene for the α-subunit of *Methanobacterium formicicum* FDH, a cysteine-containing homologue of the selenopolypeptide of FDH_H (Zinoni *et al.*, 1987). This was achieved firstly by changing the cysteine codon into UGA, and secondly, by introducing an mRNA secondary structure downstream of the UGA which closely resembled that identified for *fdhF* mRNA (Heider and Böck, 1992).

The aim of this study was to determine the detailed structural requirements within the *fdhF* mRNA that are responsible for the specific incorporation of selenocysteine. The data indicate the existence of a recognition sequence located 3′ of the UGA codon which (i) precludes recognition of the UGA as a termination signal, and (ii) directs selenocysteine insertion, most probably by the existence of a SELB/selenocysteyl-tRNA/GTP ternary complex preformed on the mRNA.

Results

Requirements for codon–anticodon interaction

Stop codon recognition in *E.coli* involves the action of the two release factors RF-1 and RF-2. While recognition of UAG is an exclusive feature of RF-1, UGA stop codons are recognized solely by RF-2 and UAA codons by both RF-1 and RF-2 (reviewed by Tate *et al.*, 1990). Since only UGA codons are so far known to be used for selenocysteine incorporation, RF-2 could possibly exert a specific function in this process. It was examined, therefore, whether it is possible to direct selenocysteine incorporation by the other stop codons, UAG and UAA.

Plasmid pFM323 contains 59 nucleotides around the selenocysteine codon (UGA_{140}) of *fdhF* cloned in-frame into the *lacZ* gene (Zinoni *et al.*, 1990); the UGA_{140} codon was changed into UAG and UAA by site-directed mutagenesis (Figure 1A). Complementary changes were introduced into the anticodon of $tRNA^{Sec}$, namely from UCA to CUA and UUA (Figure 1B). Single copies of mutated *selC* genes were then introduced at the λ-*att* site of the chromosome of a Δ*selC* strain, as described by Baron *et al.* (1990). The resulting strains contain the same *selC* fragment as strain WL81460/*selC*2013 which harbours an integrated wild-type *selC* gene (Baron *et al.*, 1990). These strains were transformed with plasmids carrying the constructed gene fusions and selenocysteine incorporation into fusion proteins was determined.

Table I shows the effects on readthrough over the different 'stop' codons measured by β-galactosidase activity. the 'wild-type' UGA codon can only be decoded by wild-type $tRNA^{Sec}$; whereas the UAG and UAA codons are solely recognized by $tRNA^{Sec}$ variants containing mutated anticodons. The highest readthrough was obtained with the wild-type UGA/UCA pair, followed by the UAA/UUA and UAG/CUA pairs. Interestingly, a third position 'wobble' interaction seems to allow the decoding of UAA and UAG codons by each of the mutated anticodons, with the UAG/UUA pair at a reduced rate and to a marginal extent with the UAA/CUA pair (Table I).

A [^{75}Se]selenite labelling experiment proved that the readthrough values measured via β-galactosidase activity corresponded to the incorporation of selenocysteine into the fusion proteins. As shown in Figure 2, the amount of ^{75}Se-labelled polypeptides paralleled the readthrough values shown in Table I. In addition, the incorporation of [^{75}Se] into the 110 kDa selenopolypeptide of FDH_O (Sawers *et al.*, 1991) was undetectable when the anticodon of $tRNA^{Sec}$ was mutated to UUA or CUA (Figure 2).

Requirements for specific recognition of selenocysteine codons

Since the UGA codon directing selenocysteine insertion could be replaced by a number of other codons without loss of coding capacity, there must be additional mRNA elements that determine its specificity. Indeed, it has been shown

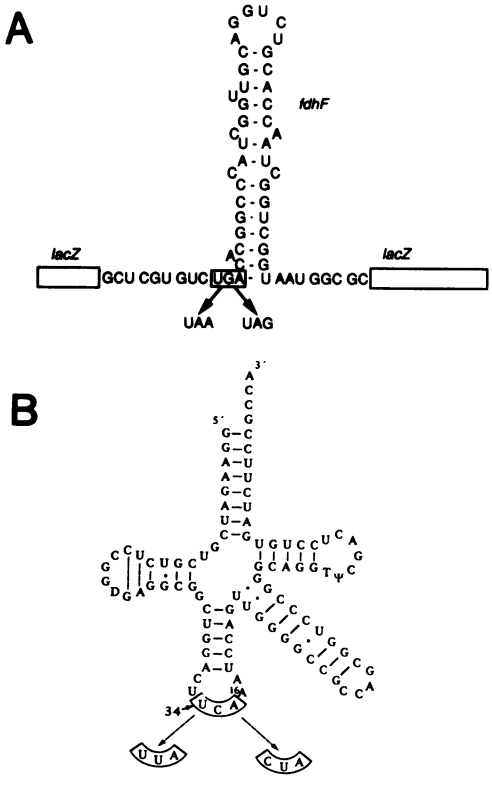

Fig. 1. Selenocysteine codon and anticodon mutations. (**A**) *fdhF* mRNA secondary structure in plasmid pFM323 and codon mutations generated. (**B**) Structure of $tRNA^{Sec}$ and anticodon mutants constructed.

Table I. Readthrough analysis with *fdhF–lacZ* gene fusions containing either of the three stop codons at the site of the selenocysteine codon of *fdhF*. Values are given in units according to Miller (1972)

Anticodon	Codon		
	UGA	UAA	UAG
UCA	3970	<1	<1
UUA	2	3240	1515
CUA	1	168	2685

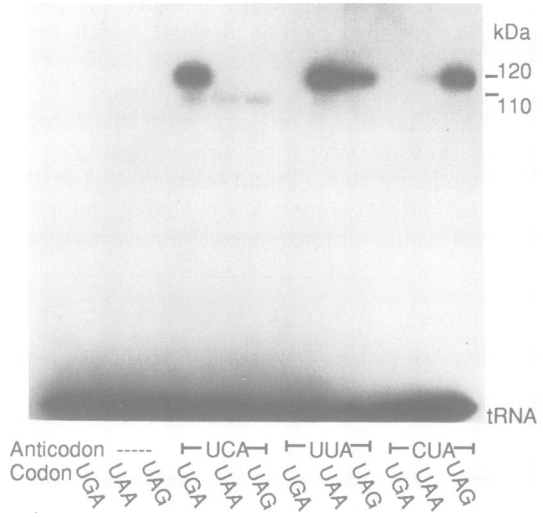

Fig. 2. [^{75}Se]selenite incorporation directed by 'stop' codons. Cells of WL81460 (Δ*selC*) and WL81460 carrying wild-type $selC_{UCA}$, $selC_{UUA}$ and $selC_{CUA}$ were tranformed with plasmids carrying *fdhF–lacZ* gene fusions containing UGA, UAA and UAG at the site of the selenocysteine codon and grown aerobically in the presence of [^{75}Se]selenite. An autoradiogram of a 10% SDS–polaycrylamide gel after electrophoretic separation of whole cell extracts is shown. The migration positions of the product of the gene fusion (120 kDa), of the selenopolypeptide of FDH_O (110 kDa) and of tRNAs are indicated. The anticodons of the $tRNA^{Sec}$ species and the codons of the gene fusions present in the strains are indicated.

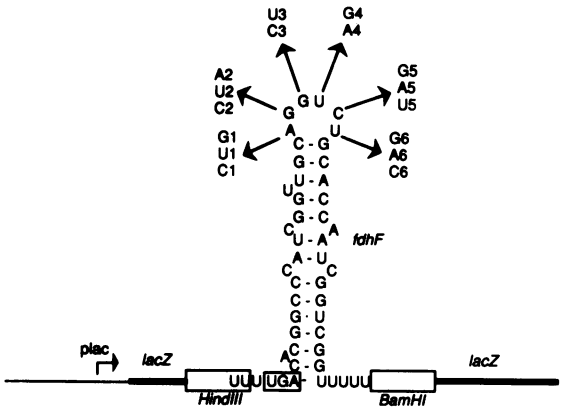

Fig. 3. *LacZ* gene fusions containing a DNA cartridge out of *fdhF*, shown as RNA in the predicted stem−loop structure. The single base mutations introduced in the loop region are indicated.

previously that a stem−loop structure immediately 3′ of the UGA_{140} (selenocysteine) codon of *fdhF* mRNA is very likely to contain additional determinants (Zinoni *et al.*, 1990). To resolve the sequence determinants within this secondary structure which are required for directing selenocysteine insertion, we have introduced single base changes in the region immediately downsteam of the UGA codon. To this end, a 'minimum cartridge' out of the *fdhF* gene consisting of the TGA_{140} codon and the 39 3′-flanking nucleotides comprising the stem−loop structure of the mRNA was cloned in-frame into the *lacZ* gene. Oligo(T) sequences were added at both ends to exclude possible linker effects on the formation of the predicted mRNA secondary structure (Figure 3). Expression of the gene fusion containing this cartridge, as measured by β-galactosidase activity, was found to be almost as high (85%) as that of a similar fusion (located on plasmid pFM324) described earlier (Zinoni *et al.*, 1990) which contains one base 5′ and 47 bases 3′ of TGA_{140} out of the *fdhF* gene.

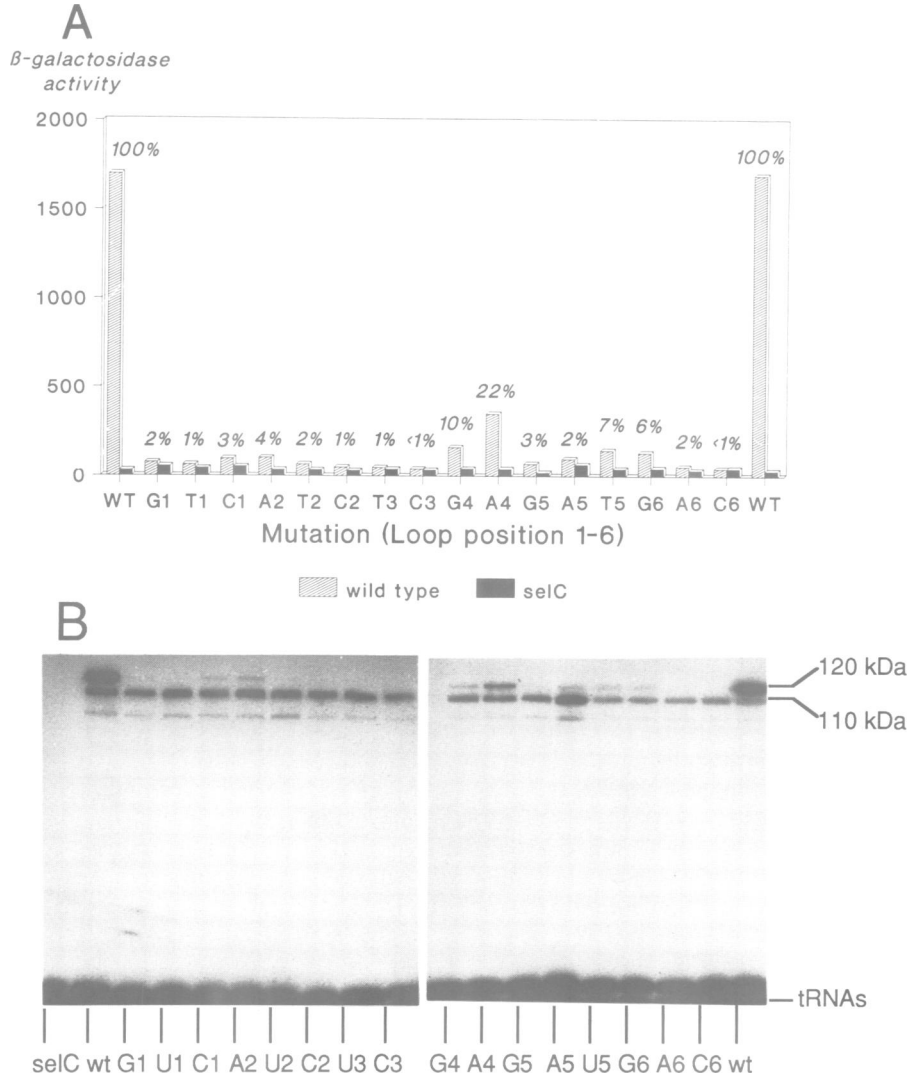

Fig. 4. Effects of single base mutations in the loop of the mRNA secondary structure on readthrough of the UGA codon for selenocysteine (**A**) and on selenocysteine incorporation into fusion proteins (**B**). The strains FM434 and FM464 were used as hosts for transformation of plasmids containing mutated *fdhF*−*lacZ* fusions. The percentage readthrough values given in (A) are corrected versus the background activities of the individual mutated fusions measured in FM464 (Δ*selC*). In (B) an autoradiogram of an 11% SDS−polyacrylamide gel is shown after electrophoretic separation of extracts from aerobically grown cells. The weak band at 95 kDa represents a degradation product of the 110 kDa selenopolypeptide of FDH_O. '*selC*' refers to strain FM464 (Δ*selC*) and 'wt' to strain FM434 (*selC*+), both containing a gene fusion with a wild-type fdhF cartridge. The mutant cartridges carried by FM434 are designated by the mutation present.

From results obtained previously during the mutagenesis of the *M.formicicum fdhA* gene (Heider and Böck, 1992) we expected the RNA loop to contain sequence information important for selenocysteine incorporation. Therefore, single mutations were first introduced into the 'minimum cartridge' that alter the nucleotide sequence of the RNA loop (Figure 3). The mutations were assayed for their effects on readthrough over the UGA codon. Figure 4A shows that all 16 mutations tested resulted in drastically decreased readthrough. The values correlated well with reduced selenocysteine incorporation into the fusion proteins, as is evident from ^{75}Se incorporation experiments (Figure 4B).

The contribution of the helical region of the stem−loop structure to specific recognition of the UGA codon was determined by analysing the effect of mutations that should affect the sequence or conformation of the secondary structure. First, the two base pairs adjacent to the RNA loop were altered such that the stability of the stem−loop stucture was predicted to stay constant (Figure 5A). Inversion of the C−G base pair immediately adjacent to the RNA loop into G−C resulted in a decrease in expression of the corresponding gene fusion to 30%, whereas an inversion of the second G−C base pair to C−G did not change the level of expression (Figure 5B). Thus, the complete RNA loop plus the adjacent base pair seem to be important. Mutations that preclude the formation of these two base pairs (Figure 5A) completely abolished *selC*-dependent readthrough of the fusions (structures S14 and S23, Figure 5B). The read-

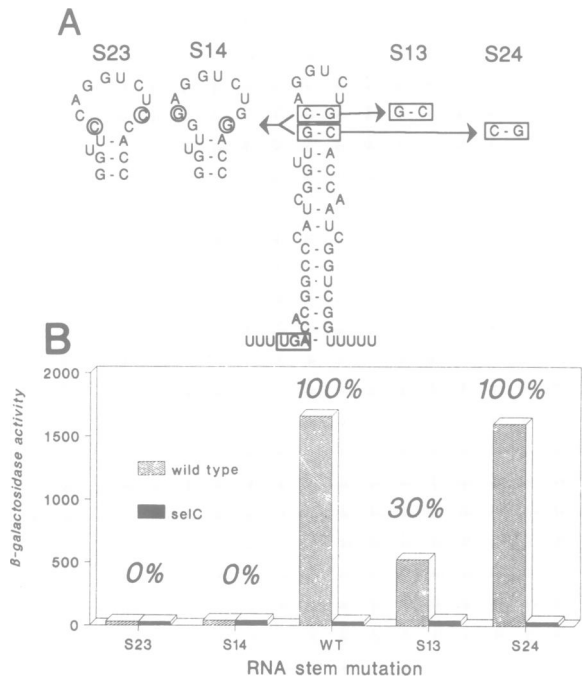

Fig. 5. Effects of mutations in the base-paired region of the mRNA secondary structure. (**A**) Mutations introduced into the stem−loop structure. (**B**) Readthrough values of *lacZ* fusions containing mutated *fdhF* cartridges in FM434 (*selC*$^+$) and FM464 (Δ*selC*).

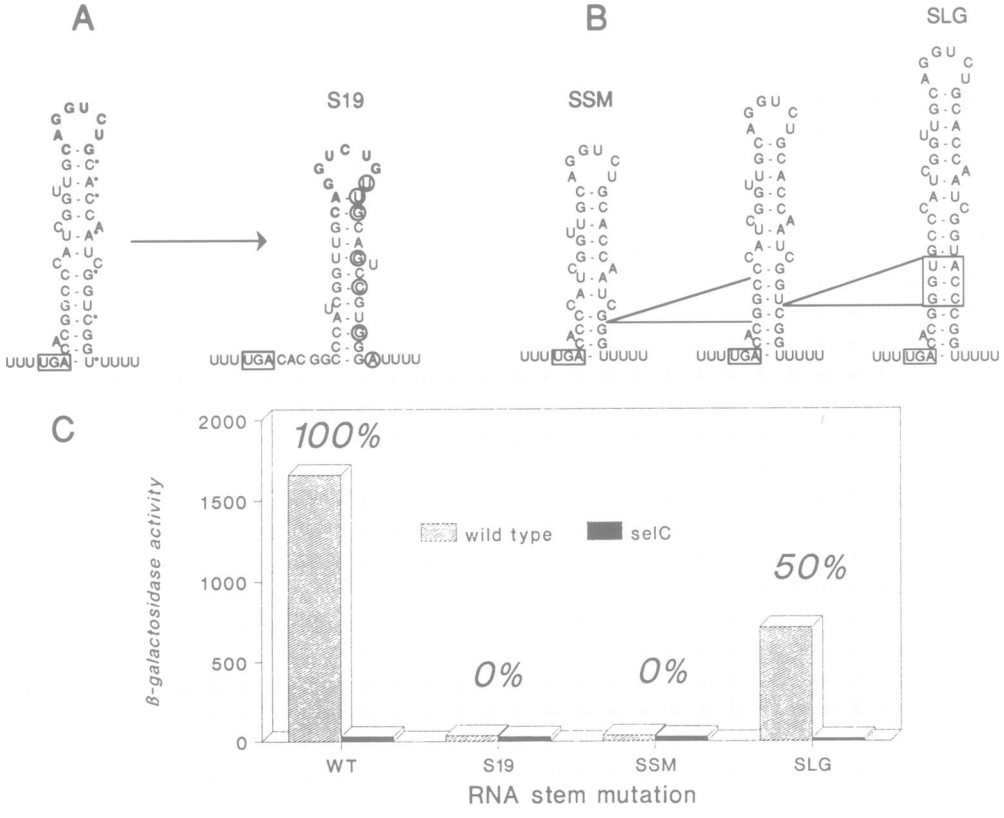

Fig. 6. Mutations affecting the conformation and the length of the mRNA secondary structure. (**A**) Effect of the introduction of several changes into the distal part of the RNA stem (indicated by asterisks) on the formation of possible secondary structures. (**B**) Effect of altered conformation on readthrough values in FM434 (*selC*$^+$) and FM464 (Δ*selC*). (**C**) Effect of shortening and extending the stem of the mRNA secondary structure. (**D**) Readthrough of *lacZ* fusions containing *fdhF* cartridges with shortened and extended secondary structures.

through values obtained by measuring β-galactosidase activity were again confirmed by [^{75}Se]selenium incorporation assays into the fusion proteins (Figure 7).

The importance of the existence of a recognition sequence in the correct secondary structural position was also tested by mutating several bases in the distal arm of the stem−loop structure. The mutations were introduced just 3′ to the RNA loop nucleotides, outside of the region defined above as being responsible for selenocysteine incorporation. The mutated cartridge is predicted to form another secondary structure with the UGA moved away from the helical part (Figure 6A). Analysis of the *lacZ* fusion obtained revealed that no *selC*-dependent β-galactosidase activity (Figure 6C) and no incorporation of [^{75}Se]selenium can be detected (Figure 7). Clearly, the change in mRNA conformation abolishes recognition of UGA as a selenocysteine codon although the loop sequence was not altered.

Finally, the influence of the length of the stem−loop structure was investigated. Two mutations were introduced, either deleting (SSM; Figure 6B) or adding three base pairs (SLG; Figure 6B) at the bottom of the RNA stem. The shortened version resulted in a complete loss of readthrough activity (Figure 6C), consistent with the previously reported absence of selenocysteine incorporation into the product of a mutated *M.formicicum fdhA* gene harbouring a shortened stem−loop structure (Heider and Böck, 1992). Surprisingly, however, readthrough was observed with a gene fusion containing a 3 bp longer stem−loop structure, albeit reduced to 50% in comparison with that promoted by the wild-type structure (Figure 6C). Again, these results were confirmed by incorporation of [^{75}Se]selenite into the fusion proteins (Figure 7).

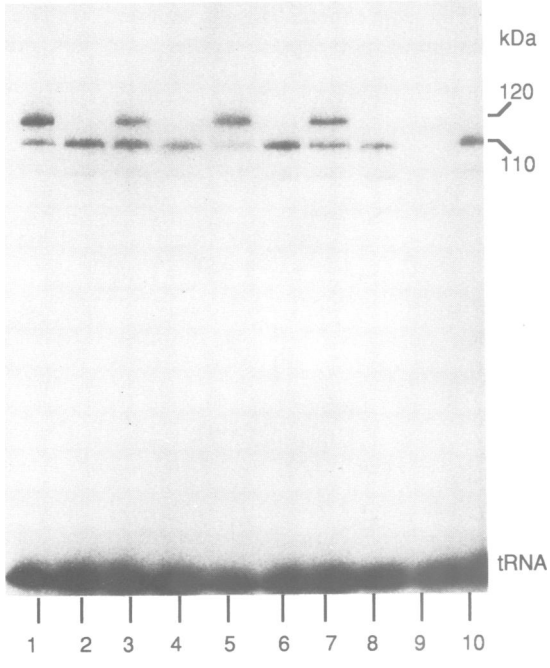

Fig. 7.]^{75}Se]selenite incorporation into proteins directed by gene fusions containing mutations in the base-paired region of the *fdhF* mRNA secondary structure. An autoradiogram of an 11% SDS−polyacrylamide gel is shown, in which extracts of aerobically grown cells transformed with plasmids containing mutated *fdhF−lacZ* gene fusions have been separated. Lane 1, FM434 carrying the wild-type fusion; lane 2, mutation S14; lane 3, S13; lane 4, S23; lane 5, S24; lane 6, S19; lane 5, SLG; lane 8, SSM; lane 9, FM464 carrying the wild-type fusion; and lane 10, FM434 carrying a gene fusion with a +1 frameshift on plasmid p+1.

Requirement of reading frame maintenance

Recognition systems for 'special' mRNA contexts in *E.coli* have previously been known for some 'programmed' frameshift events, like the +1 frame shift required for translation of the RF-2 mRNA (Weiss *et al.*, 1988) or −1 frameshift events in the translation of the *dnaX* and *insAB* mRNAs (Tsuchihashi and Kornberg, 1990; Sekine and Ohtsubo, 1989). This prompted the analysis of whether the specific recognition mechanism of selenocysteine codons is able to suppress frameshift mutations. To achieve this, *fdhF* cartridges were cloned into *lacZ* which place the UGA codon for selenocysteine into reading frames +1 and −1 by adding or deleting one of the U residues in the oligo(U) stretch at the 5′-flank of the UGA codon (Figure 8). Analysis of β-galactosidase activity produced by the resulting gene fusions on plasmids p+1 and p−1 revealed that neither of the frameshift mutations were corrected by insertion of selenocysteine. The readthrough values obtained were the same in *selC*$^+$ and Δ*selC* strains (Table II) and no selenocysteine incorporation into a fusion protein was detectable (Figure 7 for p+1). Obviously, the selenocysteine codon needs to be in the right reading frame to be decoded correctly. Interestingly, a rather high level of *selC*-independent frameshift suppression was detected with the +1 frameshift mutation when compared with the levels measured with the −1 frameshift mutation (Table II), probably because the five contiguous Us present in p+1 represent a more efficient 'shifty sequence' (Atkins *et al.*, 1990).

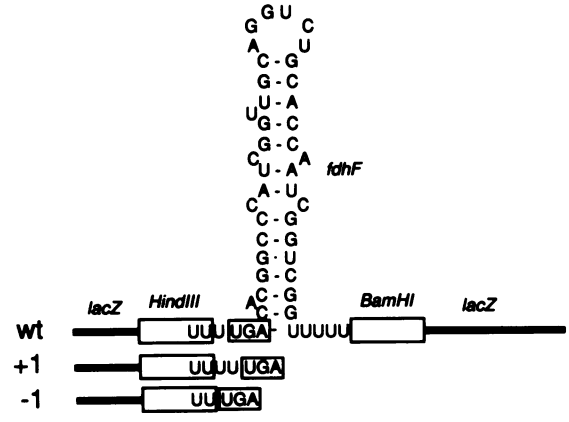

Fig. 8. Sequence of the *fdhF* cartridges containing −1 and +1 frameshift mutations.

Table II. Readthrough analysis with *fdhF−lacZ* gene fusions containing frameshift mutations immediately upstream of the selenocysteine codon of *fdhF*. Values are given in Miller units

	FM434 (selC$^+$)	FM464 (ΔselC)
pWT	1700	39
p+1	59	60
p−1	8	10

Discussion

The results presented demonstrate that three requirements in the mRNA of the *E.coli* FDH_H selenopolypeptide must be fulfilled to obtain selenocysteine incorporation into protein.

(i) Codon–anticodon interaction must follow the rules of the genetic code. An unusual 'four-way wobble' relationship at the third position of the codon seems to be operative with wild-type $tRNA^{Sec}$ which recognizes the cysteine codons UGC and UGU almost as well as the UGA codon (Baron *et al.*, 1990). This is consistent with the presence of an unmodified U at position 34 (the first position of the anticodon) of this tRNA (Schön *et al.*, 1989). The same situation seems to exist with the mutant $tRNA^{Sec}$ species containing the anticodons UUA and CUA. They can decode either UAG or UAA codons with selenocysteine, but the C^{34} present in $tRNA_{CUA}$ restricts 'wobble' base pairing much more than the U^{34} in $tRNA_{UUA}$ (Table I). Similar situations of 'four-way wobble' through unmodified U^{34} residues are only known for mitochondrial tRNAs (Jukes, 1990).

The existence of a 'third-position wobble' indicates that selenocysteine incorporation is an 'A-site' event at the ribosome, in contrast to the situation in the other known translation events which depend on codon context, like translation initiation and programmed frameshift events. These are 'P-site' events with the possibility for 'first-position wobble' interactions (Weiss *et al.*, 1989).

None of the tRNA mutants with altered anticodons showed unspecific incorporation of selenocysteine into their proteins, nor was any effect detected on the viability of strains harbouring these tRNAs. Thus, although $tRNA^{Sec}$ competes efficiently for particular UGA codons with other tRNAs or with release factors, it is obviously unable to compete for standard codons.

(ii) The selenocysteine codon has to be in the correct reading frame; this requirement can be deduced from the fact that the incorporating system does not possess 'framing' activity.

(iii) Codons determining selenocysteine incorporation must be flanked by a specific recognition sequence. This sequence consists of a stem–loop structure in the selenoprotein mRNA whose loop portion acts as the main recognition element. The secondary structure plus this recognition element were shown to be the only determinants required for incorporation. Long-range interactions, e.g. formation of an RNA pseudoknot, as required for the signalling of many −1 frameshifting events (Atkins *et al.*, 1990) do not seem to be involved.

Single base changes in the RNA sequence of the loop dramatically decrease selenocysteine incorporation. The extent of the decrease, however, was dependent on the position of the base change within the loop. Whereas selenocysteine incorporation is abolished by the C3 mutation, it is still functioning at 20% of the wild-type level with mutation A4 (Figure 3).

What may be the function of this 'recognition element'? The most plausible role is that it interacts with some other components of the translational machinery which could be either some component of the ribosome, or the translation factor SELB itself.

A very attractive candidate for the specificity factor is the selenocysteine–$tRNA^{Sec}$ specific translation factor SELB. SELB differs from EF-Tu mainly in its larger size due to a C-terminal extension. This additional domain does not exhibit any homology with translation factors and could be responsible for additional functions of this protein, e.g. the specific recognition of the mRNA context. Some indications for a direct involvement of SELB in the recognition of selenocysteine codons are being investigated by the generation of second-site reversion mutants in *selB* reverting the effects of one of the RNA loop mutations described in this communication (A.Herzog, C.Baron and A.Böck, unpublished).

From the available data the model shown in Figure 9 can be proposed for specific selenocysteine incorporation. Specific binding of Sec–$tRNA^{Sec}$ and of GTP to SELB have already been shown by Forchhammer *et al.* (1989). We suggest specific binding of the SELB protein to the secondary structure of the mRNAs for selenoproteins. A ternary complex between SELB/GTP/Sec–$tRNA^{Sec}$ would then bind to the mRNA immediately downstream of selenocysteine codons. Movement of the UGA_{140} codon into the ribosome would lead to melting of the basal part of the hairpin, leaving the upper 3 bp still closed when the UGA arrives at the A site. It is postulated that under this condition the selenocysteyl–tRNA carried by SELB is drawn into the decoding site, thus delivering the tRNA into the correct position for codon–anticodon pairing. After selenocysteine is incorporated, the ribosome would have to displace SELB/GDP from the mRNA to finish synthesis of the selenopolypeptide.

Formation of this complex can provide a biochemical basis for most of the experimental results reported. (i) The lack of termination of polypeptide synthesis at this mRNA

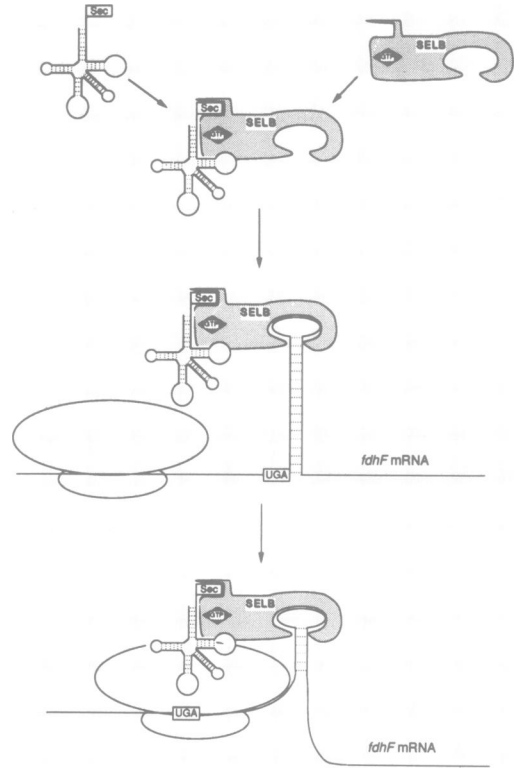

Fig. 9. Schematic model for the site-specific translational incorporation of selenocysteine into protein. The oval structures depict the translating ribosome.

position could be caused by steric hindrance by SELB of the binding of release factors. (ii) The binding of ternary complexes to mRNA results in the increase of the local concentration of the cognate aminoacyl−tRNA which may also favour competition with non-cognate tRNAs. For efficient competition it is obvious that the selenocysteyl−tRNA in the ternary complex must be provided at the correct geometrical distance. Shortening the helical part of the structure could thus lead to incorrect positioning of the anticodon relative to the codon occupying the A site whereas elongation may allow insertion due to some flexibility of the structure.

At present, we cannot exclude the possibility that the single base mutations introduced in the experiments reported could result in changes in the RNA secondary structure. For example, possible alternative foldings of the wild-type secondary structure, yielding derivatives with a more 'closed' loop (a tetra- or even a di-loop) (Cheong *et al.*, 1990) should be affected by most of the mutations.

A certain degree of variation of the recognition element is evident from the sequence of the gene for the 110 kDa selenopolypeptide of FDH_N (Berg *et al.*, 1991a). A stem−loop structure very similar to that of *fdhF* mRNA has been identified as a determinant of selenocysteine incorporation in the mRNA for this selenoprotein (Berg *et al.*. 1991b) which displays some differences in its loop region, compared with *fdhF*, namely the formation of a seven-membered loop and a one-nucleotide deviation.

Materials and methods

Bacterial strains and plasmids

E.coli JM109 (r_{K12}^-, m_{K12}^+ *recA*) (Yanisch-Perron *et al.*, 1985) was used for constructing and recovering plasmids for sequencing. Strain CJ236 (*dut, ung*) (Kunkel *et al.*, 1987) was used for site-directed mutagenesis in M13 vectors. Strains FM434 (*rpsE*-13, *selC*$^+$, F′:*lacI*) and FM464 (FM434. Δ*selC*) (Zinoni *et al.*, 1990) were employed as isogenic strain couples for analysing the *selC*-dependent readthrough of UGA codons and selenocysteine incorporation into fusion proteins. Strain WL81460 (*rpsE*13, Δ*selC*) (Zinoni *et al.*, 1990) was used as host for inserting single copies of mutated *selC* genes into the chromosome as described previously (Baron *et al.*, 1990), using a λ-phage-based insertion system (Simons *et al.*, 1987). For mutagenesis via M13 phages, appropriate restriction fragments from *fdhF* or *selC* were cloned into M13mp18 or M13mp19 (Yanisch-Perrron *et al.*, 1985). Plasmids pSKS106 (Shapira *et al.*, 1983) and pFM 323 (Zinoni *et al.*, 1990) were used for creating cartridge constructs of wild-type and mutant *fdhF* sequences inserted in-frame into *lacZ*. For initial cloning and screening of the cartridges, plasmids pUC9 (Vieira and Messing, 1982) or pT3T7lac (Boehringer Mannheim) were used.

Recombinant DNA techniques

Standard techniques were applied as described in Maniatis *et al.* (1982). DNA fragment recovery from agarose gels was done according to Vogelstein and Gillespie (1979) and transformation was performed as described by Hanahan (1985). The dideoxynucleotide chain termination method (Chen and Seeburg, 1985) was employed for DNA sequencing, using T7 polymerase (Tabor and Richardson, 1987) and synthetic oligonucleotide primers.

Mutagenesis techniques

Mutated DNA cartridges were generated *in vitro* by annealing either two or four oligonucleotides, filling in single-stranded regions and cloning the products into pUC9 or pT3T7lac. In the case of the mutageneis of the loop of the predicted RNA secondary structure, six separate fill-in reactions were performed. In each of the reactions one of the six different oligonucleotides, each degenerate at one position of the loop, was used. It was annealed with three oligonucleotides providing the rest of the cartridge such that the site of degeneracy was situated in a single-stranded region. The two oligonucleotides lying within the annealed product had been phosphorylated at their 5′-ends. The annealed product was treated simultaneously with T4 DNA polymerase and T4 DNA ligase to allow formation of double-stranded DNA cartridges. In the case of the mutagenesis of base-paired regions of the predicted RNA secondary structure oligonucleotides were annealed pairwise and filled using Klenow enzyme. In both cases the products were cleaved with *Hin*dIII and *Bam*HI at restriction sites provided at their ends and cloned into pUC9 or pT3T7lac. After screening by sequencing, the wild-type and mutant cartridges were cloned into pSKS106 and rechecked by sequencing.

Frameshift mutations were obtained as cloning artefacts during the screening of mutations by sequencing. The +1 and −1 frameshift mutations were combined with wild-type 3′-sequences using a *Bsp*MI site located in the respective cartridges. Codon and anticodon mutations were generated using the M13 mutagenesis system of Kunkel *et al.* (1987).

Assay of β-galactosidase activity

Transformed cells were grown aerobically in medium containing 1% tryptone, 0.5% yeast extract, 0.5% glycerol, 100 mM potassium phosphate (pH 7.0), 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 50 μg/ml ampicillin, 1 mM IPTG, 1 μM Na_2SeO_3 and the trace elements according to Neidhardt *et al.* (1974) and chilled on ice when an A_{600} of 0.4 was reached. β-Galactosidase activity was assayed in triplicate as described by Miller (1972). All activities were determined at least twice independently. Values are given in 'Miller units'. Relative readthrough values are corrected for *selC*-independent readthrough which was measured in parallel.

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

Cells transformed with plasmids containing gene fusions were grown aerobically to an A_{420} of 1.0 in the presence of 0.5 μM [^{75}Se]selenite in the medium described above. Whole cell lysates were subjected to SDS−PAGE and the selenopolypeptides were visualized by autoradiography (Cox *et al.*, 1981).

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References

Atkins,J.F., Weiss,R.B. and Gesteland,R.F. (1990) *Cell*, **62**, 413−423.
Baron,C., Heider,J. and Böck,A. (1990) *Nucleic Acids Res.*, **18**, 6761−6766.
Berg,B.L., Li,J., Heider,J. and Stewart,V. (1991a) *J. Biol. Chem.*, **266**, 22380−22385.
Berg,B.L., Baron,C. and Stewart,V. (1991b) *J. Biol Chem.*, **266**, 22386−22391.
Böck,A., Forchhammer,K., Heider,J. and Baron,C. (1991) *Trends Biochem. Sci.*, **16**, 463−467.
Chen,E.Y. and Seeburg,P.H. (1985) *DNA*, **4**, 165−170.
Cheong,C., Varani,G. and Tinoco,I.,Jr (1990) *Nature*, **346**, 680−682.
Cox,J.C., Edwards,E.S. and DeMoss,J.A. (1981) *J. Bacteriol.*, **145**, 1317−1324.
Ehrenreich,A., Forchhammer,K., Tormay,P., Veprek,B. and Böck,A. (1992) *Eur. J. Biochem.*, **206**, 767.
Förster,C., Ott,G., Forchhammer,K. and Sprinzl,M. (1990) *Nucleic Acids Res.*, **18**, 487−491.
Forchhammer,K. and Böck,A. (1991) *J. Biol. Chem.*, **266**, 6324−6328.
Forchhammer,K., Leinfelder,W. and Böck,A. (1989) *Nature*, **342**, 453−456.
Forchhammer,K., Leinfelder,W., Boesmiller,K., Veprek,B. and Böck,A. (1991) *J. Biol. Chem.*, **266**, 6318−6323.
Hanahan,D. (1985) In Glover,D. (ed.), *DNA Cloning*. IRL Press, Oxford, Vol. 1, pp. 109−135.
Heider,J. and Böck,A. (1992) *J. Bacteriol.*, **174**, 659−663.
Jukes,T.H. (1990) *Experientia*, **46**, 1149−1157.
Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367−382.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Miller,J.F. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Neidhardt,F.C., Bloch,P.L. and Smith,D.F. (1974) *J. Bacteriol.*, **119**, 736−747.

Sawers,G., Heider,J., Zehelein,E. and Böck,A. (1991) *J. Bacteriol.*, **164**, 1324–1331.
Schön,A., Böck,A., Ott,G., Sprinzl,M. and Söll,D. (1989) *Nucleic Acids Res.*, **17**, 7159–7165.
Sekine,Y. and Ohtsubo,E. (1989) *Proc. Natl. Acac. Sci. USA*, **86**, 4609–4613.
Shapira,S.K., Chou,J., Richaud,F.V. and Casadaban,M.C. (1983) *Gene*, **25**, 71–82.
Simons,R.W., Houman,F. and Kleckner,N. (1987) *Gene*, **53**, 85–96.
Tabor,S. and Richardson,C.C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4767–4771.
Tate,W.P., Brown,C.M. and Kastner,B. (1990) In Hill,W. *et al.* (eds), *The Ribosome: Structure, Function and Evolution*. Am. Soc. Microbiol., Washington, DC, pp. 393–401.
Tsuchihashi,Z. and Kornberg,A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 2516–2520.
Veres,Z., Tsai,L. Scholz,T.D., Politino,M., Balaban,R.S. and Stadtman,T.C. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 2975–2979.
Vieira,J. and Messing,J. (1982) *Gene*, **19**, 259–268.
Vogelstein,B. and Gillespie,D. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 615–619.
Weiss,R.B., Dunn,D., Dahlberg,A.E., Atkins,J.F. and Gesteland,R.F. (1988) *EMBO J.*, **7**, 1503–1507.
Weiss,R.B., Dunn,D., Shuh,M., Atkins,J.F. and Gesteland,R.F. (1989) *New Biol.*, **1**, 159–169.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–109.
Zinoni,F., Birkmann,A., Leinfelder,W. and Böck,A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3156–3160.
Zinoni,F., Heider,J. and Böck,A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 4660–4664.

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