Mutagenesis of *selC*, the gene for the selenocysteine-inserting tRNA-species in *E.coli*: effects on *in vivo* function

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ABSTRACT

The selenocysteine-inserting tRNA ($tRNA^{Sec}$) of *E. coli* differs in a number of structural features from all other elongator tRNA species. To analyse the functional implications of the deviations from the consensus, these positions have been reverted to the canonical configuration. The following results were obtained: (i) inversion of the purine/pyrimidine pair at position 11/24 and change of the purine at position 8 into the universally conserved U had no functional consequence whereas replacements of U9 by G9 and of U14 by A14 decreased the efficiency of selenocysteine insertion as measured by translation of the *fdhF* message; (ii) deleting one basepair in the aminoacyl acceptor stem, thus creating the canonical 7 bp configuration, inactivated $tRNA^{Sec}$; (iii) replacement of the extra arm by that of a serine-inserting tRNA abolished the activity whereas reduction by 1 base or the insertion of three bases partially reduced function; (iv) change of the anticodon to that of a serine inserter abolished the capacity to decode UGA_{140} whereas the alteration to a cysteine codon permitted 30% read-through. However, the variant with the serine-specific anticodon efficiently inserted selenocysteine into a gene product when the UGA_{140} of the *fdhF* mRNA was replaced by a serine codon (UCA). Significantly, none of these changes resulted in the non-specific incorporation of selenocysteine into protein, indicating that the mRNA context also plays a major role in directing insertion. Taken together, the results demonstrate that the 8-basepair acceptor stem and the long extra arm are crucial determinants of $tRNA^{Sec}$ which enable decoding of UGA_{140} in the *fdhF* message.

INTRODUCTION

selC was identified previously as one of four genes whose products are required for the formation of selenoproteins in *E. coli* (1). *selC* codes for a tRNA species ($tRNA^{Sec}$) which is aminoacylated with L-serine by seryl tRNA-ligase (2) and which then serves as the substrate for selenocysteine synthase (the *selA* gene product). The latter enzyme contains covalently bound pyridoxal 5-phosphate and, via dehydration of the seryl moiety and subsequent addition of reduced selenium provided by the activity of the *selD* gene product (3) converts seryl-$tRNA^{Sec}$ into selenocysteyl-$tRNA^{Sec}$ (K.Forchhammer, K.Boesmiller, B.Veprek and A.Böck, unpublished information). In addition, insertion of selenocysteine into protein requires the function of a selenocysteyl-$tRNA^{Sec}$-specific translation factor, SELB (4, 5). The biochemical role of SELB is considered to be alternate to that of elongation factor Tu (EF-Tu) since EF-Tu binds selenocysteyl-$tRNA^{Sec}$ with a drastically reduced affinity compared to other aminoacyl-tRNAs (6).

Both, the sequence of $tRNA^{Sec}$ and its modification pattern are unique (2, 7). It deviates in several positions from the consensus hitherto considered to be invariant in elongator tRNA species (8). These deviations include: (i) an 8 bp aminoacyl acceptor stem, (ii) a purine residue at position 8 and a purine-pyrimidine pair at positions 11/24; (iii) pyrimidines at positions 14 and 15; (iv) an anticodon (UCA) matching the UGA termination triplet. Analysis of the *selC* sequence from *Proteus vulgaris* showed that these unique features are conserved (9); moreover, a compensatory nucleotide exchange at positions 14/21 in the *Proteus* (C/G) relative to the *E. coli* (U/A) sequence indicated the existence of an extended D-stem region in this tRNA (see Fig. 1).

These unique and conserved primary and secondary structural features might be involved in any one of the following functions of $tRNA^{Sec}$: (i) interaction with the biosynthetic enzyme, selenocysteine synthase, which forms a specific and stable complex with seryl-$tRNA^{Sec}$ (K.Forchhammer and A.Böck, unpublished data); (ii) interaction with translation factor SELB which forms a complex with selenocysteyl-$tRNA^{Sec}$; (iii) preclusion of interaction with EF-Tu. In order to gain information on the particular functions of these unique structural elements we have constructed mutant $tRNA^{Sec}$ derivatives in which each one of the non-canonical positions was reverted to the consensus. Moreover, we have changed the anticodon to sense codons. The

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in vivo functional consequences on UGA read-through and selenoenzyme formation are reported.

MATERIAL AND METHODS

Strains, media and plasmids

The bacterial strains and plasmids used in this work are listed in Table 1. Strain WL81932 was constructed by phage P1-mediated transduction of Δ*fdhF-mel*::Tn*10* from strain FM932 into strain WL80460. Transductants were cured of the Tn*10* (10) and the *recA* mutation harboured by strain JC10289/pKY102 was subsequently introduced by P1 transduction. LB medium (11) was used for aerobic growth of bacteria. For anaerobic cultivation TGYEP-medium (12) was employed which was supplemented with 0.1% (w/v) formate [for β-galactosidase and formate dehydrogenase H (FDH_H) assays] or with 1% (w/v) nitrate [for formate dehydrogenase N (FDH_N) assays].

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

^{75}Se incorporation experiments were carried out as described by Cox *et al.* (13). [^{75}Se]selenite was added to the medium at a final concentration of 1 μM and at a specific radioactivity of 150 to 1,500 μCi/μmol. Cell lysates were separated in SDS polyacrylamide gels (12.5%).

Enzyme assays

β-Galactosidase activity was determined as described by Miller (11). FDH_H assays were performed as described by Sawers *et al.* (14) and FDH_N activity was measured according to Ruiz-Herrera *et al.* (15). Values given are the average of two to four independent determinations.

Recombinant DNA techniques

Standard recombinant DNA techniques were employed as described by Maniatis *et al.* (16). Restriction fragments were recovered after size fractionation in agarose gels by adsorption to glass powder (17). Transformation of bacteria was accomplished as given by Chung *et al.* (18).

Table 1. Strains and plasmids

E. coli Strains	Relevant Genotype	Reference
FM433	Δ(*argF-lac*)U169 *rpsE13* Δ(*srl-recA*)306::Tn*10*	(22)
WL81460	like FM433 Δ(*selC*)400	(22)
WL81460-SelC	lambda lysogenic derivates of WL81460 carrying chromosomally integrated *selC* genes (wild-type and mutants); for nomenclature see Fig. 1	this work
WL80460	like WL81460 *recA*$^+$	W.Leinfelder
JC10289	Δ(*srl-recA*)306::Tn*10*	(27)
FM932	Δ*fdhF*-mel::Tn*10*	F.Zinoni
WL81932	like WL81460 Δ*fdhF*-mel::Tn*10*	this work
WL81932-SelC -SelC/TGA -SelC/GCA	lambda lysogenic strains carrying *selC* genes with altered anti-codon	this work
JM109	Δ(*lac-proAB*) F'[*traD36 proAB* lacI^q *lacZ* ΔM15]	(24)
CJ236	*dut-1 ung-1 thi-1 relA1*/pCJ105	(19)
Plasmids		
pBFM20	Ap^R *fdhF* (TGA)*	this work
pBFM201	Ap^R *fdhF* (TGC)*	this work
pBFM202	Ap^R *fdhF* (TGT)*	this work
pBFM203	Ap^R *fdhF* (TCA)*	this work

*Letters in parentheses refer to codon 140 of *fdhF* in different plasmids, respectively.

Site directed mutagenesis of the *selC* gene

Oligonucleotide directed mutagenesis was performed essentially as described by Kunkel *et al.* (19). For this purpose a 233 bp *Xho*II-fragment from plasmid pMN81 (2) carrying the wild-type *selC* gene was cloned into *Bam*HI-digested vector M13mpl9. Screening for mutant clones was carried out via DNA sequencing (20).

The mutated genes *selC*ExB and *selC*ExBK were obtained by a different procedure. Plasmid pCB3 was constructed by ligation of the 233 bp *Xho*II fragment from pMN81 with *Bam*HI linearized vector pUC19. After restriction of the resulting plasmid with *Sau*96I (cutting twice in the extra arm region) and fill-in with Klenow fragment the resulting pieces of the *selC* gene were ligated with *Bam*HI 8-mer linkers. Both pieces of the *selC* gene were either directly re-ligated after *Bam*HI restriction (*selC*ExB) or after fill-in (*selC*ExBK). The authenticity of all constructs was verified by DNA sequencing.

Chromosomal integration of *selC* genes

The mutant *selC* genes were integrated into the chromosome in order to compare the complementing activity to that of the wild-type gene; the lambda-based integration system described by Simons *et al.* (21) was employed. Since the *selC* deletion strain WL81460 (22) used as host for integration was kanamycin resistant the original selection system for recombinant phage integration had to be modified.

For that purpose plasmid pRS552 (21) was linearized by *Eco*RV restriction and ligated with a 1.2 kb *Hin*PI fragment from plasmid pACYC184 (23) carrying the chloramphenicol resistance gene. The resulting plasmid pRSC was checked by restriction analysis to ensure that transcription of the resistance gene was directed away from that of the inserted *selC* gene.

Wild-type and mutant *selC* genes were cloned via polylinker sites from M13mpl9 into pUC19 (24) and finally ligated with *Bam*HI linearized plasmid pRSC. Resulting plasmids were controlled by DNA sequencing; chloramphenicol-resistant strains carrying chromosomally integrated recombinant phages were selected after recombination with lambda RS45 (21).

RESULTS

Construction of mutant *selC* genes

Figure 1 shows the nucleotide sequence of *selC* from *E. coli* and displays the mutational alterations introduced into it; they can be grouped into four classes:

Class I comprises mutant tRNAs in which base substitutions have been introduced into the D-stem/loop region: G8 has been 'reverted' to the canonical T8; T9 and T14 have been altered into G9 and A14, respectively, and the G11/C24 pair has been inverted to the canonical pyrimidine/purine (C11/G24) configuration. A T8A14 double mutant gene was also constructed.

Class II mutant tRNAs have an altered extra arm. The ExB version is one base shorter (21 nucleotides) and its distal sequence of 7 bases was changed; ExBK contains an elongated version of the extra arm (25 nucleotides) and ExS contains the extra arm of a serine cognate tRNA ($tRNA_{UGA}$).

Class III mutant tRNAs contain a shortened aminoacyl-acceptor stem. The A5a/T67a pair has been deleted in delAc; intermediates

in construction of these mutant gene have either A5a(del5′) or T67a(del3′) deleted.

In class IV mutants the anticodon (TCA) has been changed into serine (TGA) and cysteine (GCA) specific anticodons.

The authenticity of all mutant tRNA genes constructed has been verified by DNA sequencing. With the aid of the integration system described by Simons *et al.* (21), the wild-type *selC* gene and the mutant variants were integrated into the λ attachment site of the chromosome from strain WL81460 which carries a deletion of the *selC* gene. By Southern hybridisation analysis of chromosomal DNA of the resulting lysogens it was demonstrated that a single copy of the *selC* variants had been integrated (data not shown). It is emphasized that all *selC* genes (except WL81460−2013) are preceded by exactly the same 70 bp 5′ flanking sequence indigenous to wild-type *selC* (2).

Effect of the *selC* mutations on UGA_{140} read-through

To measure the efficiency of the various mutant tRNAs in decoding UGA_{140} of the *fdhF* mRNA, plasmid pFM320 was transformed into the lysogens. pFM320 carries a *fdhF* cartridge comprising 67 bp upstream and 47 bp downstream of TGA_{140} cloned int thσ 5′ portion of *lacZ* (22). The gene fusion is under the control of the *lac* operon promoter. The lysogens were grown anaerobically and β-galactosidase activity was measured (Fig. 2). The T8 and the 11/24 mutations reduce read-through only marginally, if at all. On the other hand, A14 and G9 mutant gene products are definitely less efficient; the concomitant presence of the T8 plus A14 changes consistently alleviated the restriction afforded by A14 alone.

The most dramatic effects were observed when deletions were introduced into the acceptor stem of $tRNA^{Sec}$. A reduction to the canonical 7 bp length, similar in sequence to cognate serine inserting tRNA, completely abolished UGA_{140} read-through. Surprisingly, replacement of the extra arm with that of a serine inserter tRNA also was detrimental to the function. Reduction by one basepair (ExB) caused a suppression of read-through to about 1/3 of that observed with wild-type $tRNA^{Sec}$ whereas an increase by 3 bp had no effect at all.

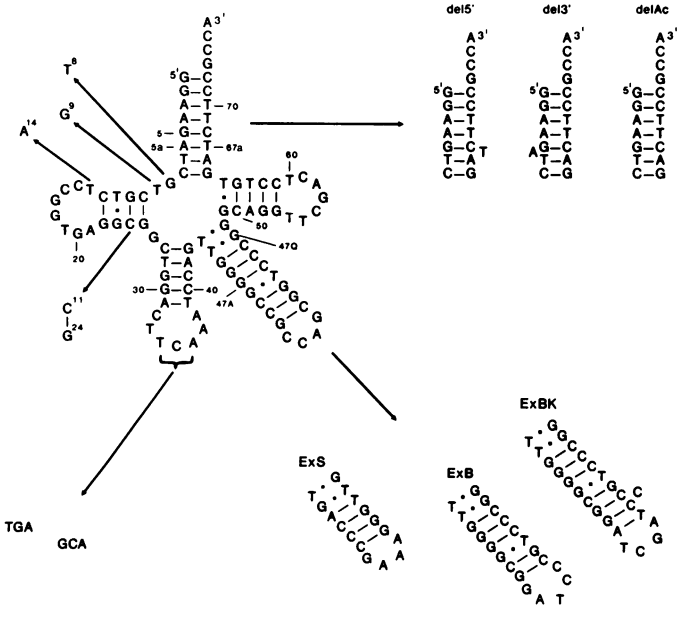

Figure 1. Nucleotide sequence of *selC* and of the mutational derivatives constructed. Alterations introduced are indicated by arrows. The numbering is according to Sprinzl *et al.* (8); nucleotide positions in the acceptor stem that were deleted in this work are marked as 5a/67a. The designations used for the different *selC* alleles (e.g. T8, del3′) are indicated.

Effect of *selC* mutations on the synthesis of formate dehydrogenase selenopolypeptides

E. coli possesses two formate dehydrogenases, FDH_H and FDH_N, both of which contain selenopolypeptide subunits (13, 25): FDH_H has an 80 kDa selenopolypeptide whereas the size of the FDH_N selenoprotein is 110 kDa. FDH_H is induced under fermentative conditions whereas FDH_N induction requires anaerobiosis plus nitrate as external electron acceptor (13, 25).

The effect of the various mutant $tRNA^{Sec}$ species on the formation of FDH_H and FDH_N was of interest since it was thought to provide a more realistic picture of their functional proficiency than the pFM320-based system; the selenopolypeptides are encoded by single genes whereas the *lac* fusions of pFM320 are present on a multicopy plasmid and they are under the control of a strong promoter. The resulting abundance of mRNA in the latter system may not give a true representation of the consequences to the natural system of any functional deficiency of the *selC* mutants. Moreover, read-through, as measured by FDH formation, is determined in the homologous mRNA context. Figure 3A presents the results of FDH_H activity assays which show that these considerations indeed hold true. Although the overall tendency is the same, the effects of the G9 and A14 changes are less severe. An exception is the ExBK variant of *selC* which promoted complete UGA read-through in the *lacZ*-based system but not in the natural environment of UGA_{140}. Again, the *selC* variants with deletions in the aminoacyl acceptor stem are inactive.

Figure 3B gives the values for FDH_N. They resemble the results of the FDH_H measurements, except those of the extra arm mutants. FDH_N formation is clearly more sensitive to alterations in this part of the molecule.

Selenium incorporation experiments

As a direct biochemical measure of the *in vivo* function the incorporation of [^{75}Se]selenium into selenoproteins directed by

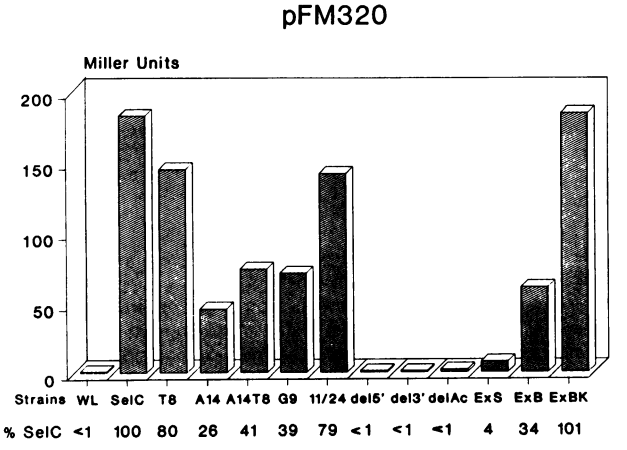

Figure 2. Effects of *selC* mutations on UGA decoding. Lysogenic derivates of the *selC* deletion strain WL81460 (WL) carrying chromosomally integrated *selC* genes (wild-type and mutant *selC* genes like T8, A14 etc.) were transformed with plasmid pFM320 (22). UGA decoding was monitored by measuring β-galactosidase activity in cultures that were grown anaerobically in TGYEP medium (pH 6.5) to which formate was added at 0.1% (w/v). For nomenclature of mutant derivates, see Figure 1.

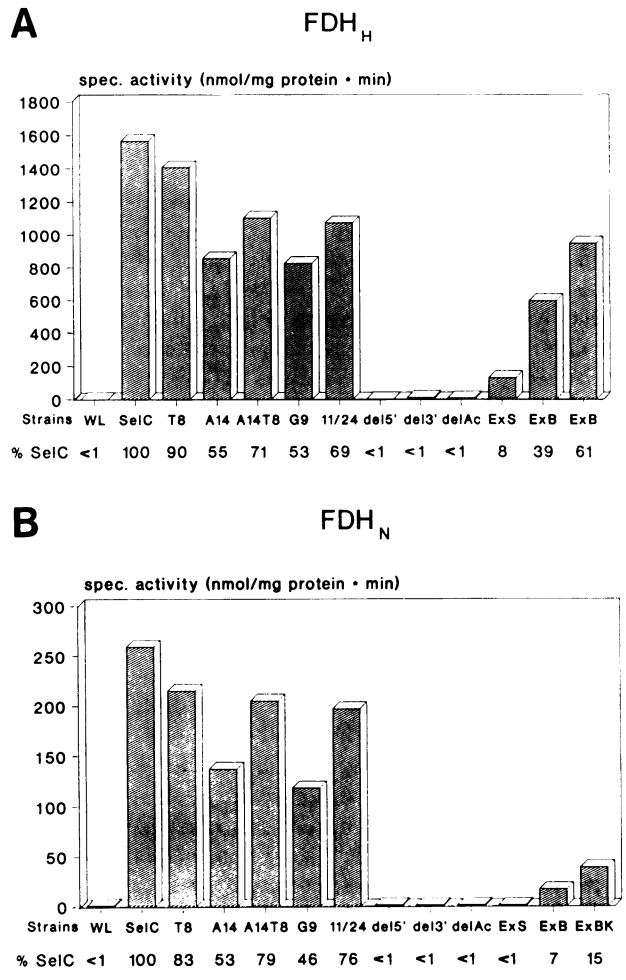

Figure 3. Effects of *selC* mutations on formation of FDH_H **(A)** and FDH_N **(B)** enzyme activity. Enzyme activity was determined from extracts of cells that were grown anaerobically in TGYEP medium (pH 6.5) to which either formate was added at 0.1% (w/v) (A), or nitrate at 1% (w/v) (B). Nomenclature of lysogenic strains is as described in Figure 1.

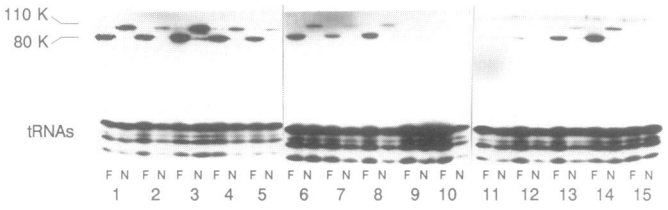

Figure 4. ^{75}Se incorporation into FDH_H (80 K) and FDH_N (110 K) selenopolypeptides directed by mutant tRNAs. An autoradiograph of cell lysates separated by SDS-PAGE is shown. Cells were grown anaerobically in TGYEP-medium supplemented with formate (F) at 0.1% (w/v) or nitrate (N) at 1% (w/v). Control strains FM433 $selC^+$ (lane 1) and *selC* deletion strain WL81460 (lane 15). Strains WL81460-SelC (lane 2) and WL81460−2013 (lane 3) have chromosomally integrated wild-type *selC* genes which differ in the length of the upstream region (70 and 200 bp, respectively). Mutated *selC* genes are integrated in strains WL81460-T8 (lane 4), -Al4 (lane 5), -A14T8 (lane 6), -G9 (lane 7), −11/24 (lane 8), -del5′ (lane 9), -del3′ (lane 10), -delAc (lane 11), -ExS (lane 12), -ExB (lane 13) and -ExBK (lane 14).

the various mutant tRNAs was determined. Fig. 4 shows that the incorporation pattern correlates with the results of the read-through analysis (Fig. 2) and of FDH activity determinations

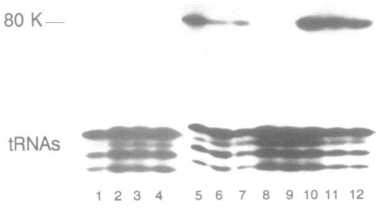

Figure 5. ^{75}Se incorporation into FDH_H selenopolypeptides directed by $tRNA^{Sec}$ variants mutated in the anticodon. Plasmids pBFM20 (TGA_{140}), pBFM201 (TGC_{140}), pBFM202 (TGT_{140}) and pBFM203 (TCA_{140}) were constructed by ligation of the 3.5 kb *ScaI*/*Hin*dIII fragments of plasmids pFM20−203 (26), carrying *fdhF* genes, with pBR322 restricted with *Hin*dIII and *Eco*RV. Strain WL81932 (Δ*selC*, Δ*fdhF*) transformed with plasmid pBFM20 (lane 1), pBFM201 (lane 2), pBFM202 (lane 3) and pBFM203 (lane 4). Strain WL81932 carrying chromosomally integrated wild-type *selC* gene and transformed with plasmid pBFM20 (lane 5), pBFM201 (lane 6), pBFM202 (lane 7) and pBFM203 (lane 8). Strain WL81932 carrying chromosomally integrated *selC* with a TGA anticodon and transformed with pBFM20 (lane 9) and pBFM203 (lane 10); strain WL81932 carrying chromosomally integrated *selC* with a GCA anticodon and transformed with pBFM20 (lane 11) and pBFM201 (lane 12).

Table 2. *In vivo* effects of anticodon changes in $tRNA^{Sec}$ on UGA decoding capacity

Strains	Translational *lacZ* fusion (pFM320)	Enzyme Activity FDH_H	FDH_N
WL81460			
-SelC/TCA (Sec)*	100%	100%	100%
-SelC/TGA (S)	<1%	<1%	<1%
-SelC/GCA (C)	36%	32%	28%

*Sec, S and C denote anticodons specific for selenocysteine, serine and cysteine codons, respectively.

(Fig. 3). The mutations in the D-stem/loop region only quantitatively affect selenopolypeptide formation whereas changes in the aminoacyl acceptor helix and shortening of the extra arm are detrimental. Two facts are noteworthy. First, none of the mutant *selC* tRNAs promoted Se incorporation into any polypeptide other than the FDH subunits, i.e. incorporation was specific in all cases. Secondly, a comparison of the labelling pattern of strain WL81460-SelC and WL81460−2013 demonstrates that the latter construct exhibits a more intensive incorporation. Both strains carry the wild-type form of *selC* but WL81460−2013 contains a 200 bp long 5′ flanking region compared to the 70 bp of WL81460-SelC and of all other integrated *selC* genes. This indicates that *selC* is expressed at a lower level in the constructs with the shorter 5′ flanking region which is desirable for the present work since partial functional deficiencies are not masked by a surplus of the respective gene product.

Alterations of the anticodon

To analyse the contribution of the $tRNA^{Sec}$ anticodon to the specificity of UGA-directed selenocysteine insertion we have constructed two derivatives with anticodons specific for serine (UGA) and cysteine (GCA). The altered genes were integrated into the chromosome of strain WL81460 (Δ*selC*) and the efficiency of their gene products to function in UGA decoding was measured with the aid of the translational *lacZ* fusion carried by plasmid pFM320 and by determination of FDH activity (Table 2). The results show that conversion to a cysteine-specifying anticodon allows substantial read-through and FDH formation,

whereas the serine derivative was inactive. This result correlates closely with previous findings, namely that conversion of TG-A_{140} into TCA_{140} (serine) of the *fdhF* gene abolished selenocysteine incorporation whereas changes into cysteine codons (TGT and TGC) allowed substantial insertion in a *selC*$^+$ genetic background (26).

The *selC* genes harbouring the mutated anticodons were tested for their capacity to insert selenocysteine into gene products derived from *fdhF* genes containing TGA, TGC (cysteine) and TCA (serine) codons in position 140 (Fig. 5). The $tRNA^{Sec}$ derivative containing the cysteine-specific anticodon inserted selenocysteine directed by the UGA and UGC codons, whereas the variant with the serine anticodon only determined selenocysteine insertion when position 140 of the *fdhF* mRNA was occupied by the serine (UCA) codon. Incorporation occurred solely into the *fdhF* gene product, i.e. these tRNAs do not recognize any other cysteine or serine codon of the cell.

DISCUSSION

The conservation of the non-canonical sequence features of $tRNA^{Sec}$ had led to the suggestion (9) that they either play a role in interaction with selenocysteine synthase and/or translation factor SELB or in the preclusion of interaction with EF-Tu. To differentiate between these possibilities we have 'reverted' each of these positions or structural elements into the canonical state. In addition, the size of the extra arm of $tRNA^{Sec}$ was altered and the anticodon was changed into serine and cysteine specific ones. The *in vivo* function of the respective mutant $tRNA^{Sec}$ variants was measured; it must be emphasized that the assays employed do not allow the differentiation between deficiency in charging with L-serine, binding to the biosynthetic enzyme or to SELB. Nevertheless, some important conclusions can be drawn from the results and hints could be gathered as to which $tRNA^{Sec}$ mutant should be chosen for a more detailed biochemical analysis.

A first and important conclusion was that none of the *selC* mutants displayed any unspecific incorporation of selenocysteine into protein. This means that none of the changes introduced into $tRNA^{Sec}$ provided the capability to interact with EF-Tu. It does not preclude the possibility, however, that these positions indeed are involved but only in combination with other unique features of the tRNA. This argument holds not only for the A14 and G9 mutations which showed a quantitative reduction of $tRNA^{Sec}$ efficiency but also for the 11/24 inversion and T8 transversion which left the tRNA fully functional. The partial impairment of the function in the case of the former two mutants may be caused by kinetic impairment of charging, selenocysteine synthesis or binding to SELB.

The 8 basepair aminoacyl acceptor stem is of crucial importance to the function as a selenocysteine inserter. Reduction to the canonical 7 bp length abolished read-through and selenocysteine insertion to below detectable levels. A further *in vitro* analysis has shown recently that this tRNA can still be charged with L-serine and can serve as a substrate for selenocysteine synthase, although at a reduced rate, but that it is unable to interact with SELB (Ch. Baron and A. Böck, unpublished results). It will be interesting to study its interaction with EF-Tu since it is probable that one of the structural features preventing interaction with EF-Tu is the extended aminoacyl acceptor helix. If such an interaction indeed occurs it may be feasible to achieve selenocysteine insertion at selected positions of a polypeptide chain by simply changing the anticodon of that $tRNA^{Sec}$ variant in a way that enables interaction with the codon at the respective position.

Change of the anticodon of $tRNA^{Sec}$ from UCA into cysteine (GCA) and serine (UGA) anticodons did not interfere with the charging of the mutant variants with L-serine and with formation of selenocysteine. Remarkably, selenocysteine insertion occurred only at the specific site and not at any other serine or cysteine specifying codons. Specificity, therefore, is determined primarily by the mRNA structure (22) irrespective of the nature of the codon/anticodon pairing. This result also emphasizes the lack of interaction between selenocysteyl-$tRNA^{Sec}$ and EF-Tu and the importance of the alternate elongation factor SELB for the decoding process.

Incorporation of selenium into the 110 kDa subunit of FDH_N was considerably more sensitive to some structural alterations (e.g. change of the extra arm; see Fig. 3B and 4) than that into the 80 kDa selenopolypeptide of FDH_H. It also appears that a higher level of selenocysteyl-$tRNA^{Sec}$ is required for synthesis of the 110 kDa selenopolypeptide than for that of the 80 kDa species (Fig. 4, lanes 2 and 3). It will be interesting to compare the mRNA context around the UGA codon of the FDH_N selenopolypeptide with that of the *fdhF* mRNA to gain information on the structural basis of the differential response.

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