Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4660–4664, June 1990
Biochemistry

Features of the formate dehydrogenase mRNA necessary for decoding of the UGA codon as selenocysteine

(UGA coding/mRNA structure)

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Communicated by Thressa C. Stadtman, April 10, 1990

ABSTRACT **The *fdhF* gene encoding the 80-kDa selenopolypeptide subunit of formate dehydrogenase H from *Escherichia coli* contains an in-frame TGA codon at amino acid position 140, which encodes selenocysteine. We have analyzed how this UGA "sense codon" is discriminated from a UGA codon signaling polypeptide chain termination. Deletions were introduced from the 3′ side into the *fdhF* gene and the truncated 5′ segments were fused in-frame to the *lacZ* reporter gene. Efficient read-through of the UGA codon, as measured by β-galactosidase activity and incorporation of selenium, was dependent on the presence of at least 40 bases of *fdhF* mRNA downstream of the UGA codon. There was excellent correlation between the results of the deletion studies and the existence of a putative stem–loop structure lying immediately downstream of the UGA in that deletions extending into the helix drastically reduced UGA translation. Similar secondary structures can be formed in the mRNAs coding for other selenoproteins. Selenocysteine insertion cartridges were synthesized that contained this hairpin structure and variable portions of the *fdhF* gene upstream of the UGA codon and inserted into the *lacZ* gene. Expression studies showed that upstream sequences were not required for selenocysteine insertion but that they may be involved in modulating the efficiency of read-through. Translation of the UGA codon was found to occur with high fidelity since it was refractory to ribosomal mutations affecting proofreading and to suppression by the *sup-9* gene product.**

The incorporation of selenocysteine into selenoproteins is directed by an in-frame UGA codon (for review, see ref. 1). In the case of the selenopolypeptide subunit of formate dehydrogenase H (FDH_H) (*fdhF* gene product) from *Escherichia coli* it has been shown that the insertion of this nonstandard amino acid into the growing polypeptide chain occurs cotranslationally (2). The incorporation is dependent on the presence of a unique tRNA species (the *selC* gene product), whose anticodon UCA is complementary to the UGA nonsense codon (3). This tRNA is aminoacylated with L-serine and the L-seryl-$tRNA_{UCA}$ is subsequently converted into selenocysteinyl-$tRNA_{UCA}$ by the catalytic action of two proteins, the *selA* and *selD* gene products (4, 5). Delivery of selenocysteinyl-$tRNA_{UCA}$ to the ribosome requires a protein (SelB) that specifically binds the charged tRNA (but not seryl-$tRNA_{UCA}$) and guanine nucleotides and that is considered to act as an elongation factor alternate in its function to elongation factor Tu (6).

The present paper deals with the central question as to how the UGA codon of the *fdhF* mRNA (UGA_{140}) is discriminated by the ribosome from a chain-termination UGA codon. Evidence is presented that demonstrates that the coding specificity of the UGA_{140} codon depends on the presence of, minimally, 40 bases of the mRNA immediately downstream of the UGA codon.

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MATERIALS AND METHODS

Strains, Media, and Growth Conditions. The strains of *E. coli* used in this work are listed in Table 1. The buffered rich medium used was TGYEP (pH 6.5) (8), unless otherwise stated; it was supplemented with 5 μM Na_2SeO_3 and 5 μM Na_2MoO_4. Anaerobic cultures were grown in gas-tight serum bottles under a N_2 atmosphere (9). Glycerol was added to 0.5% (wt/vol), and galactose was added to 0.4% (wt/vol) final concentration.

***In Vivo* Labeling of Selenopolypeptides with [^{75}Se]Selenite.** ^{75}Se incorporation experiments were carried out as originally described by Cox *et al.* (10). [^{75}Se]Selenite (sodium salt) was added to the medium to a final concentration of 1.5 μM and at a specific radioactivity of 210 μCi/μmol (1 Ci = 37 GBq). Cell extracts were separated in 0.1% $NaDodSO_4$/polyacrylamide gels (7% or 8%).

β-Galactosidase Assays. For the determination of β-galactosidase activity, cultures were grown at 37°C to an optical density (A_{600}) of 0.4–0.5. Enzyme activity was assayed, and the specific activity was calculated as described by Miller (11). Values given are the average of two to four independent determinations carried out in triplicate.

Recombinant DNA Techniques and Construction of *fdhF–lacZ* Fusions and of *fdhF* Cartridges. Basic recombinant DNA techniques were carried out as described by Maniatis *et al.* (12). The fusion junction of all plasmids constructed in this study were verified by DNA sequencing using the modified supercoil sequencing method (13), with T7 DNA polymerase (Pharmacia) and appropriate synthetic oligonucleotides as primers.

For generation of 3′ deletions of the *fdhF* gene, plasmid pFM22 was constructed by deletion of a 1.86-kilobase (kb) *Eco*RV/*Xba* I fragment from plasmid pFM30 (9), thereby introducing a *Bgl* II site at codon 268 of *fdhF*. Plasmid pFM22 was linearized with *Bgl* II and then treated with exonuclease BAL-31 at a degradation rate of 50 base pairs (bp)/min. After "fill-in" of 5′ protruding ends with DNA polymerase I (Klenow fragment), ligation with *Bam*HI linkers (dCGCGGATCCGCG), and restriction with *Eco*RI and *Bam*HI, the fragments were separated on a 1.5% agarose gel and recloned into *Eco*RI/*Bam*HI-digested plasmid pACYC184. Inserts were sized by restriction analysis and their exact end points were determined by DNA sequencing. These *fdhF* fragments were used to generate *lacZ* fusions with either plasmid pRS551 (14) for transcriptional or one of plasmids pFM1400, pFM1401, and pFM1402 for translational fusions. These latter three plasmids are derived from plasmids pNM480, pNM481, and pNM482, respectively (15), by replacing a 0.8-kb *Eco*RI/*Cla* I fragment of plasmid pMC1403 (16) with

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Table 1. *E. coli* strains used in this study

Strain	Relevant genotype	Ref.
MC4100	Δ(*argF-lac*)U169 *rpsL150*	7
FM433	Like MC4100, *rpsL*$^+$ *rpsE13* Δ(*srl-recA*)306::Tn*10*	This work
WL81460	Like FM433, Δ(*selC*)400::*Kan*	This work
FM434	Like FM433, F′:*lacI*^{q}, Cm^R	This work
FM464	Like WL81460, F′:*lacI*^{q}, Cm^R	This work

Cm^R, resistance to chloramphenicol.

corresponding fragments of pNM480, pNM481, and pNM482. They carry the polylinker of plasmid pUC8 (17) in front of a truncated *lacZ* gene in all possible reading frames and differ only by 1 or 2 bases, thus minimizing effects arising from different *lacZ* linker joints. These new vectors exhibited a more uniform β-galactosidase expression than the pNM plasmid series, presumably due to smaller deviations of plasmid copy number.

For construction of *fdhF* cartridges to be cloned into the *lacZ* gene, a 270-bp *Kpn* I fragment from the *fdhF* portion of a *fdhF–lacZ* fusion carried by plasmid pMC1403 (16) (fusion point +47) was replaced with a synthetic oligonucleotide linker, thus creating a *Hin*dIII site 67 bp upstream of TGA_{140}. A 1.7-kb *Eco*RI/*Hin*dIII fragment of the resulting plasmid carrying the *fdhF* promoter and the 39 5′-terminal codons was then replaced with a 160-bp *Eco*RI/*Hin*dIII fragment from plasmid pSKS106 (16) containing the promoter and N-terminal 7 codons of *lacZ*, to create plasmid pFM320. From this plasmid a 126-bp *Hin*dIII/*Bam*HI fragment (containing 117 bp of *fdhF* DNA) was excised and replaced by synthetic double-stranded oligonucleotides to yield plasmids pFM321, pFM322, pFM323, and pFM324. The sequences of the joints fusing the N-terminal portion of *lacZ* to the *fdhF* cartridge in these plasmids were all checked by DNA sequence analysis; they are given in Table 2.

RESULTS

Involvement of the *fdhF* mRNA Context in the Translation of UGA_{140}. It was demonstrated previously that selenocysteine incorporation into the *fdhF* gene product took place even when the UGA_{140} codon had been converted into cysteine (UGU or UGC) codons. In the genetic background of the streptomycin-resistant strain MC4100 (7), this UGU/UGC-determined selenocysteine insertion amounted to 5–10% of the level directed by UGA_{140} (2). Replacement of the *rpsL150* allele of MC4100 by the wild-type *rpsL* gene increased the UGC-directed selenocysteine incorporation to grossly the same level as given by UGA_{140} (data not shown). Thus, selenocysteinyl-tRNA efficiently not only competes with release factor II for the UGA "termination" codon but also can compete with other aminoacyl-tRNAs for a "wobble" codon at this position. This observation indicates a strong influence of the mRNA context around UGA_{140}, forcing selenocysteine incorporation into this position of the polypeptide chain. The "tight" coding specificity of the UGA_{140} codon could also be demonstrated in a strain having a deletion in *selC* (gene for $tRNA_{UCA}$) and harboring the *sup-9* suppressor allele (18). Suppression of UGA_{140} only amounted to 1.9% compared to 26.7% suppression of UGA_{189} of the *lacIZ* fusion carried by F′Δ14 (19) (data not shown).

Putative Secondary Structures of the *fdhF* mRNA Around UGA_{140}. Context effects on codon recognition may involve immediately adjacent bases or mRNA secondary structure (for review, see ref. 20). We have previously noticed the occurrence of a short stem–loop structure around the UGA_{140} codon of the *fdhF* mRNA (21); Fig. 1 presents the results of an extensive, computer-assisted secondary structure search. There are three possible structures that exhibit significant stability: structures a and b (Fig. 1 *a* and *b*) contain the UGA_{140} codon in one of the loops of a doublet hairpin structure, while structure c (Fig. 1*c*) has the UGA_{140} codon at the 5′ side of a stem–loop structure.

Downstream Sequences Are Required for Decoding UGA_{140} as Selenocysteine. The functional role of the putative mRNA structures depicted in Fig. 1 was tested by generating deletion derivatives of the *fdhF* gene approaching UGA_{140} from the 3′ side and by fusing them in-frame to the *lacZ* reporter gene. The β-galactosidase activity expressed from the gene fusions can be considered as a measure of translation of the UGA_{140} codon. Fig. 2*A* shows that there is a dramatic dependence on the presence of the sequence downstream of the UGA_{140} codon for β-galactosidase formation; no translation was observed in the case of *fdhF–lacZ* fusions, which contained a 4-, 7-, and even 18-bp *fdhF* segment downstream of UGA_{140}. A low level of read-through, however, was measured when the fusion joint was located in position +27. Further addition of *fdhF* downstream sequence led to an increase of *fdhF–lacZ* expression until a plateau was reached with 46 bases downstream of UGA_{140}. This level remained

Table 2. Read-through of UGA_{140} in *fdhF* cartridges cloned into the *lacZ* gene

		β-Galactosidase activity*			
		Strain FM434 (*selC*$^+$,*lacI*^{q})		Strain FM464 (Δ*selC*,*lacI*^{q})	
Plasmid	Sequence of upstream fusion joint	Glycerol + O_2	Galactose − O_2	Glycerol + O_2	Galactose − O_2
pSKS106	—	11,322	5997	12,190	5715
pFM320	$\overline{TT\ C\ TAC}\xrightarrow{-67}$	6,144	7452	9	10
pFM321	$\overline{TTC\ TAC\ GTA\ GCC}\xrightarrow{-42}$	4,406	6108	3	7
pFM322	$\overline{TTC\ TAC\ GAC}\xrightarrow{-18}$	3,919	4842	1	4
pFM323	$\overline{TTC\ TAC\ GTA\ GCT}\xrightarrow{-9}$	4,908	6693	1	3
pFM324	$\overline{TTT\ GTT\ AAC\ TGA}\xrightarrow{-1}$	1,758	3429	5	35

The bases from the eighth codon of the *lacZ* gene carried by pSKS106 (16) are denoted by a line above the sequence, those from the 5′-terminal part of the *fdhF* cartridge are denoted by an arrow below the sequence. Numbers denote the first base of the *fdhF* cartridge relative to the T of the TGA_{140} triplet. All cartridges end with the 47th bp downstream of the TGA_{140} triplet followed by the *Bam*HI linker and the rest of the *lacZ* gene.

*Growth was in a medium containing 1% tryptone, 0.1 M potassium phosphate (pH 6.5), 1 μM sodium selenite, 10 μM sodium molybdate, and 1 mM isopropyl β-D-thiogalactopyranoside.

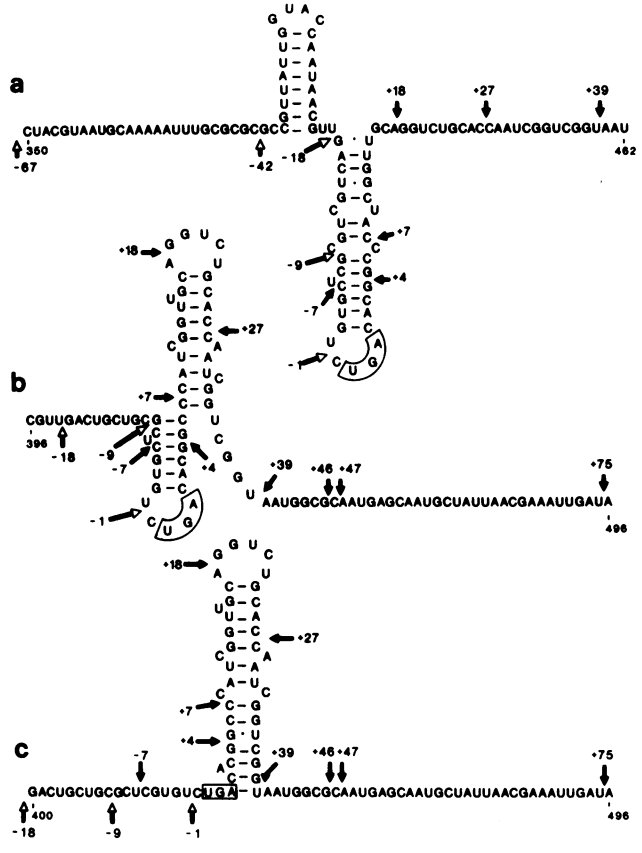

FIG. 1. Putative secondary structure of the *fdhF* mRNA segment in the vicinity of the UGA_{140} codon as suggested by the "RNA fold" program of the Genofit PC/Gene program package. Prediction is based on the method of Zuker *et al.* (22) and Jacobsen *et al.* (23). Free energy values calculated (24) for the two hairpins of structures a and b (*a* and *b*) gave values of −8.6/−10.0 and −9.6/−11.4 kcal/mol (1 cal = 4.184 J), respectively, and −17.6 for structure c (*c*). Solid arrowheads point to the end point of deletions from the 3′ side, open arrowheads denote the 5′ border of *fdhF* cartridges fused into the *Hin*dIII site of the polylinker of the *lacZ* gene from plasmid pSKS106 (see Table 2).

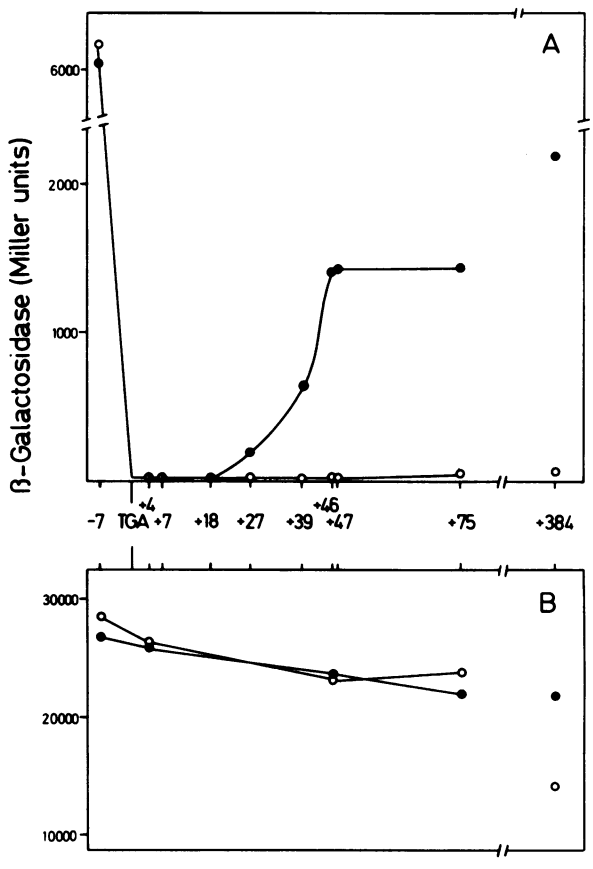

FIG. 2. Expression of β-galactosidase activity from transcriptional and translational *fdhF–lacZ* fusions. The cultures were grown anaerobically in TGYEP medium (pH 6.5). Formate was added at 30 mM to induce the *fdhF* promoter (25). The abscissa gives the location of the deletion end points upstream or downstream of the UGA (TGA) codon in *fdhF*. (*A*) β-Galactosidase activity of translational fusions, assayed in strains FM433 (*selC*$^+$) (●) and WL81460 (Δ*selC*) (○). (*B*) β-Galactosidase activity of transcriptional fusions, assayed as described above in strains FM433 (●) and WL81460 (○).

constant in the +47 and +75 fusions. The increase in expression of the +384 fusion relative to those of the +46, +47, and +75 fusions could result from translational reinitiation events far downstream of UGA_{140}.

Fig. 2 also gives the results of two important control experiments: First, there is no read-through in a strain having a defective *selC* gene product (Fig. 2*A*), which indicates that translation of the UGA_{140} codon is dependent on the presence of selenocysteinyl-$tRNA_{UCA}$. Second, it was precluded that the extremely low read-through observed for the *fdhF–lacZ* fusions with the fusion points +4, +7, and +18 in the *selC*$^+$ genetic background was the result of a strong polar effect on transcription; using plasmid pRS551, *lac* operon fusions were constructed (14) by placing 5′-terminal portions of *fdhF* (analogous to the −7, +4, +46, +75, and +384 translational fusions) in front of a *lacZ* reporter gene possessing its own translational initiation region. Termination codons in all three open reading frames located between the pRS551 polylinker and *lacZ* ensured that translation terminates downstream of the *fdhF* portion and reinitiates at the *lacZ* ribosome binding site. The results of β-galactosidase activity assays of these transcriptional fusions (Fig. 2*B*) clearly indicate the absence of strong polar effects on transcription in the vicinity of the UGA codon.

To confirm that the read-through of the UGA_{140} codon in the different *fdhF–lacZ* fusions is coupled to the insertion of selenocysteine, ^{75}Se incorporation experiments were carried out. Fig. 3 shows that there is an excellent correlation between the amount of radioactivity incorporated into the fusion proteins and the level of β-galactosidase activity. ^{75}Se incorporation into the fusion proteins also was dependent on a functional *selC* gene product (data not shown).

Upstream Sequences Are Not Required for the Specificity of UGA_{140}-Directed Selenocysteine Insertion. The secondary structure models of the *fdhF* mRNA (Fig. 1 *a* and *b*) indicate that sequences at the 5′ side of the UGA_{140} codon may participate in the formation of a stem–loop structure. It was, therefore, important to analyze whether sequences upstream of the UGA_{140} codon play a role in the efficiency and specificity of selenocysteine insertion. The strategy followed consisted of constructing *fdhF* cartridges of various lengths containing the UGA_{140} codon and inserting them into the polylinker at the 5′ terminus of the *lacZ* gene carried by plasmid pSKS106 (16). The resulting chimeric genes, therefore, are composed of the control region and the first seven codons of *lacZ*, followed by a linker sequence, a segment of variable length from the *fdhF* gene, a second linker sequence, and the downstream portion of *lacZ*. The *fdhF* cartridges differ in the *fdhF* portion upstream of UGA_{140} but they all are fused to *lacZ* 47 bp downstream of the UGA.

The β-galactosidase activities expressed from these gene fusions upon induction by isopropyl β-D-thiogalactopyranoside are given in Table 2. Read-through of UGA_{140} and selenocysteine insertion occurred under both aerobic and anaerobic conditions. When assayed in a *selC*$^+$ genetic

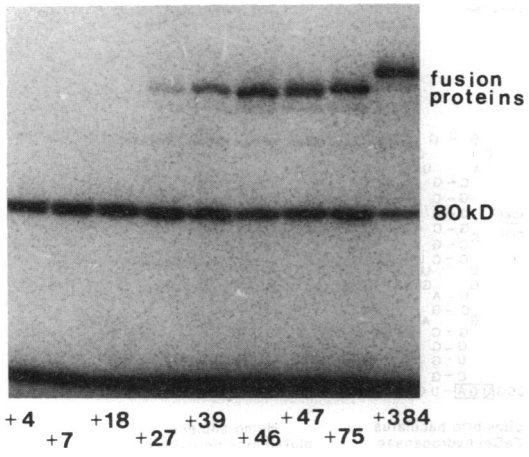

FIG. 3. Incorporation of selenium into various *fdhF–lacZ* fusion proteins. Plasmids carrying *fdhF–lacZ* fusions with the fusion joints +4, +7, +18, +27, +39, +46, +47, +75, and +384 were transformed into strain FM433, which carries an intact chromosomal *fdhF* gene that is expressed under anaerobic conditions. Autoradiograph of labeled cell extracts from anaerobically grown cells after electrophoretic separation in a $NaDodSO_4$/8% polyacrylamide gel. The sizes of the different fusion proteins vary between 131 and 145 kDa.

background there was no substantial alteration in β-galactosidase formation when the *fdhF* portion upstream of UGA_{140} codon was reduced from −67 to −9 bp. It should be noted that the −9-bp cartridge still allows the formation of the upstream hairpin structure of the putative secondary structure (Fig. 1*b*). Removal of 8 more bases consistently resulted in a quantitative reduction of read-through under all growth conditions. Moreover, a somewhat higher read-through took place in a $selC^-$ genetic background (Table 2).

The results of the assessment of read-through as measured by β-galactosidase formation were corroborated by the analysis of ^{75}Se incorporation into the fusion proteins (Fig. 4).

DISCUSSION

Incorporation of selenocysteine into proteins follows a unique pathway: First, selenocysteine is the only nonstandard amino acid known until now that is incorporated during polypeptide chain elongation at the ribosome (2). Second, the cotranslational mode of insertion follows a bypass of the classical way of protein synthesis since delivery of selenocysteinyl-tRNA to the ribosome is not by elongation factor Tu but rather by a selenocysteinyl-tRNA-specific elongation factor, the *selB* gene product (4–6). Clearly, elucidation of the mechanism of this unique process is of basic interest. It provides information on how the genetic code can be modified to accommodate information for additional amino acids. One of the most interesting questions in this respect is how the internal UGA codon is differentiated from a UGA codon signaling chain termination. Providing an answer to this question not only may contribute to our knowledge on partial reactions of peptide bond formation and chain termination at the ribosome but may ultimately provide a means of introducing targeted insertions of selenocysteine or even of nonnatural amino acids into proteins.

The results presented have shown that context-dependent incorporation of selenocysteine occurs when the UGA_{140} is converted into cysteine (UGC or UGU) codons—i.e., when a mismatch is present in the wobble position. The efficiency of this wobble interaction is reduced in strains carrying a restrictive *rpsL* allele. The tight coding specificity of UGA_{140} is also documented by the fact that there is only a low level of suppression by the *sup-9* gene product in the absence of selenocysteinyl-tRNA.

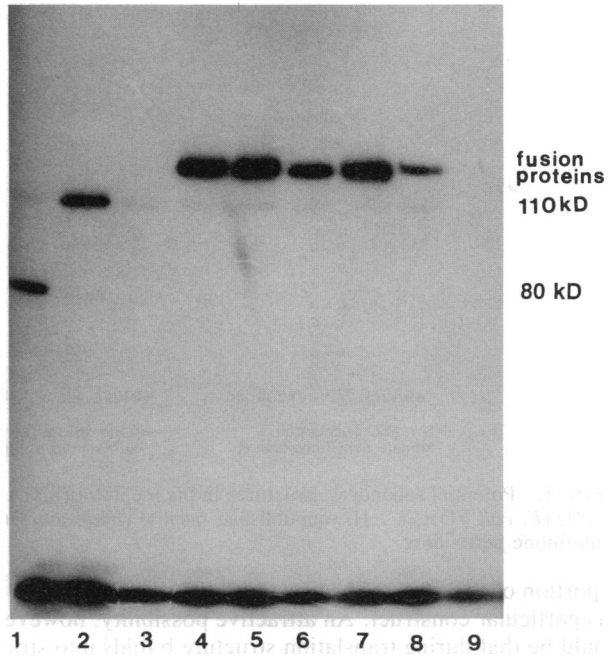

FIG. 4. ^{75}Se incorporation into products of *lacZ* genes carrying *fdhF* gene cartridges. An autoradiograph of cell lysates separated by $NaDodSO_4$/PAGE is shown. Strain FM434 ($lacI^q$) grown anaerobically in TGYEP medium without (lane 1) and with (lane 2) 1% KNO_3 as controls for intrinsic selenoproteins; strain FM434 transformed with plasmids pSKS106 (lane 3), pFM320 (lane 4), pFM321 (lane 5), pFM322 (lane 6), pFM323 (lane 4), pFM324 (lane 8); and strain FM464 (Δ*selC*, $lacI^q$) transformed with plasmid pFM324 (lane 9). Transformants were grown aerobically as described in Table 2.

Surprisingly, it turned out that the mRNA context required for selenocysteine insertion extended to >27 bases downstream of the UGA_{140} codon, with efficient read-through occurring only when the downstream DNA segment was >39 bases. This considerable stretch of mRNA is longer than that covered by the ribosome during protein chain elongation (26); it can be taken as circumstantial evidence that a mRNA secondary structure plays a crucial role in the process of UGA_{140} decoding. Indeed, the results of the 3′ deletion studies (see Fig. 2) support the notion that read-through depends on the formation of the downstream hairpin (Fig. 1*c*). Deletions that destroy this structure completely (fusion points +4, +7, and +18) prevent UGA_{140} translation.

Of particular interest are those deletions that permit only partial read-through—i.e., the deletions with fusion points +27 and +39. Surprisingly, the +39 fusion allows only 50% read-through, although the stem–loop structure is unaltered. The +27 fusion reduces read-through to ≈10% of the level displayed by the +46, +47, or +75 fusions. At the moment, we cannot preclude that the level of read-through observed with these constructs is influenced by the G+C-rich *Bam*HI linker used to fuse the *fdhF* segments to *lacZ*: In the case of the +39 fusion, this linker can extend the hairpin structure by at least 1 bp further; in the case of the +27 fusion, part of the *Bam*HI linker can base pair and lead to a partial restoration of the secondary structure.

Sequences of the *fdhF* mRNA upstream of the UGA_{140} codon are not essential for the specificity of selenocysteine insertion, but they may modulate the efficiency of the process. Removal of the upstream loop of the putative secondary structure (Fig. 1*b*) leads to a considerable reduction of β-galactosidase formation. It is, however, still open whether this decrease is due to reduced read-through of the UGA_{140} codon or, alternatively, the consequence of an overall reduction of translation due to the change introduced into the

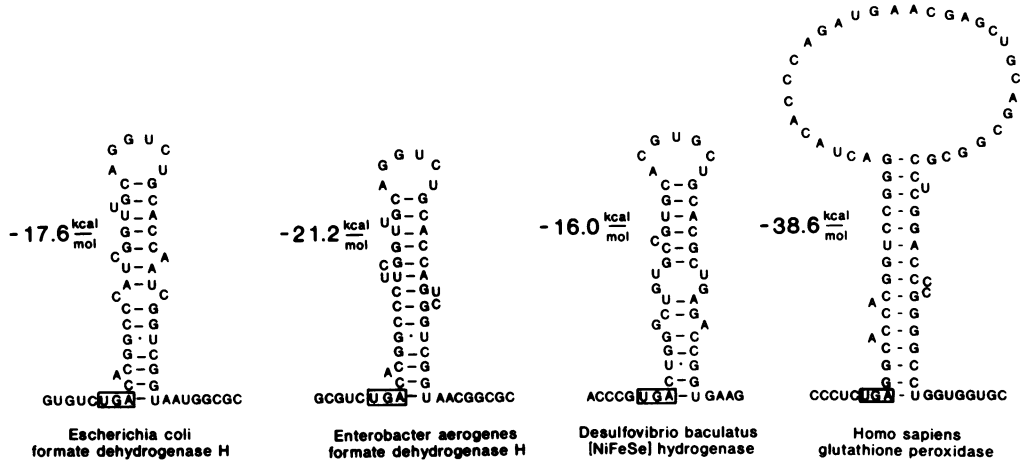

FIG. 5. Potential secondary structures in the vicinity of UGA codons in mRNAs coding for selenoproteins. Sequences are from Zinoni *et al.* (21) (*E. coli* FDH_H), J.H. (unpublished results) (*Enterobacter aerogenes* FDH_H), Menon *et al.* (27) (hydrogenase), and Ishida *et al.* (28) (glutathione peroxidase).

5′ portion of the *fdhF* mRNA by the A+T-rich linker used in this particular construct. An attractive possibility, however, would be that during translation structure b folds into structure c when a ribosome opens this upstream helix.

We have searched the available mRNA sequences encoding selenoproteins for secondary structures in order to gain phylogenetic support for a possible functional role of this mRNA region. The results shown in Fig. 5 demonstrate that similar structures can indeed be found. Whether or not they are functionally significant requires experimental proof.

There are several ways in which the secondary structure described could promote translational read-through and selenocysteine insertion. It could cause ribosomes to pause when approaching the UGA, allowing increased time for selenocysteinyl-tRNA to bind. That pausing indeed may occur can be taken from the fact that fusions that do not contain the UGA_{140} consistently show higher levels of β-galactosidase formation compared to those having the codon (see Fig. 2*A*). Another alternative might be that the secondary structure masks the UGA and prevents it from being recognized by 16S rRNA (29) and/or by release factor 2. It also cannot be ruled out at present that a particular sequence or structure of the hairpin is recognized by the *selB* gene product. Introduction of mutations into this particular region will aid in differentiating between these possibilities.

We thank M. Casadaban, J. Miller, D. Hughes, and W. Leinfelder for their generous supply of strains and plasmids. The help of G. Sawers in improving the manuscript is gratefully acknowledged. We are greatly indebted to R. Mertz and G. J. Arnold for synthesizing oligonucleotides. This work was supported by grants from the Bundesministerium für Forschung und Technologie (Genzentrum München) and the Fonds der Chemischen Industrie.

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