Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 4181–4185, May 1993
Biochemistry

Interaction of translation factor SELB with the formate dehydrogenase H selenopolypeptide mRNA

(UGA coding/RNA–protein interaction/mRNA structure)

CHRISTIAN BARON, JOHANN HEIDER, AND AUGUST BÖCK*

Lehrstuhl für Mikrobiologie der Universität München, Maria-Ward-Strasse 1a, W-8000 Munich 19, Germany

Communicated by Thressa C. Stadtman, December 22, 1992

ABSTRACT **The SELB protein from *Escherichia coli* is a specialized elongation factor required for the UGA-directed insertion of the amino acid selenocysteine into selenopolypeptides. Discrimination of the UGA codon requires the presence of a recognition element within the mRNA, which is located at the 3′ side of the UGA codon; a hairpin structure can be formed within this mRNA region. By gel shift assays, a specific interaction between SELB and the mRNA recognition element could be demonstrated. Footprinting experiments, using nucleases or iodine as cleaving agents, showed that SELB binds to the loop region of the hairpin structure. In the presence of selenocysteinyl-tRNA, SELB formed a complex with the charged tRNA and the mRNA. The results indicate that targeted insertion of selenocysteine is accomplished by the binding of the SELB protein to this mRNA recognition element, resulting in the formation of a selenocysteinyl-tRNA·SELB complex at the mRNA in the immediate neighborhood of the UGA codon.**

A number of unusual coding events, which contradict the hitherto paradigmatic scheme of protein synthesis, have been discovered during the last few years (for review, see refs. 1 and 2). Besides ribosome hopping and frameshifting, a particularly intriguing finding was that the genetic capacity of a cell can be expanded in such a way that a nonstandard amino acid, selenocysteine, is incorporated into polypeptides. This amino acid is present in several proteins from organisms belonging to all three lines of descent, archaea, bacteria, and eukarya (for review, see refs. 3 and 4). The first indication that selenocysteine is inserted cotranslationally was provided by the finding that the genes for two selenoproteins—namely, *fdhF* coding for a formate dehydrogenase from *Escherichia coli* (5) and *gpx* coding for a glutathione peroxidase from mouse (6)—contain an in-frame TGA (UGA) codon. Since then, a number of genes coding for other selenoproteins also have been found to possess an in-frame TGA (UGA) codon (7–15). With the exception of the gene for plasma selenoprotein P, which contains 10 TGA (UGA) codons, all these genes contain only 1 such codon.

A biologically basic question bearing considerable relevance for the understanding of the translation and the evolution of the genetic code is how the translational machinery can cope with the situation that one and the same triplet can signal either chain termination or selenocysteine insertion. The best-examined systems up to now are those of the *E. coli* formate dehydrogenase H (*fdhF*) and N (*fdnG*) genes (5, 10). Their analysis has revealed that a sequence of 40 bases in the mRNA at the 3′ side of the UGA codon, which can be folded into a putative hairpin structure, is required for selenocysteine insertion (16, 17). Mutagenesis of this hairpin structure showed that the sequence of the loop region is particularly important and that it may serve as a recognition element for some putative factor directing the selenocysteine-inserting tRNA species ($tRNA^{Sec}$) to decode that particular UGA (18). Similar structures are conserved in many, albeit not all, selenoprotein mRNAs (16). In mRNAs coding for mammalian selenoproteins, no consensus sequence or structure could be derived from the analysis of the context of the UGA codons. Conserved hairpin structures, however, are present in the 3′ untranslated regions, and the importance of these selenocysteine-insertion sequences (SECIS) was demonstrated (19).

A further relevant finding was that there is not an absolute requirement for a UGA codon to determine selenocysteine insertion, since this amino acid was also incorporated when the UGA was replaced by one of the other two termination codons or by a sense codon, provided that the anticodon of $tRNA^{Sec}$ was changed to match the mutated codon (18, 20). Clearly, the context of the UGA (i.e., the recognition element) is the major determinant for the specific decoding of this mRNA position.

During translation, this recognition element of the mRNA has to interact with some other macromolecular component of the translation machinery to confer the required specificity in the decoding of the UGA. Analysis of the pathway for selenocysteine biosynthesis and insertion in *E. coli* has identified an appealing candidate for such a specificity factor, the SELB protein. SELB is an elongation factor alternate in its function to elongation factor EF-Tu that transports selenocysteinyl-$tRNA^{Sec}$ to the ribosome (21–23). We demonstrate in this communication that SELB specifically binds to the loop region of the mRNA recognition element of the *E. coli fdhF* mRNA and forms a complex with selenocysteinyl-$tRNA^{Sec}$ at the mRNA. On the basis of our results, we propose a mechanism for selenocysteine insertion into proteins at specifically programmed UGA codons that involves a translation factor bound to the mRNA region 3′ to the UGA that delivers selenocysteinyl-$tRNA^{Sec}$ to the ribosomal A site.

MATERIALS AND METHODS

Preparation of 5′ Labeled RNA Transcripts. Plasmids for the generation of wild-type (wt) and C3 transcripts (see Fig. 3) were generated by ligation of DNA fragments from vectors containing selenocysteine-insertion cartridges (18) into the *Stu* I site of the T7 plasmid pET-7 (24). Transcripts were produced from *Bst*NI-linearized plasmid templates according to Wyatt *et al.* (25). Synthesis of phosphorothioate-containing transcripts was accomplished according to Schatz *et al.* (26). Transcripts were 5′ labeled with [γ-^{32}P]ATP followed by separation in denaturing polyacrylamide gels and elution (27).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: $tRNA^{Sec}$, selenocysteine-inserting tRNA; wt, wild type; CVN, cobra venom nuclease; SECIS, selenocysteine-insertion sequences.

*To whom reprint requests should be addressed.

Gel Shift Assays. A standard gel shift assay contained 1.5 pmol of transcript in buffer K (100 mM potassium phosphate, pH 7/10 mM $MgCl_2$/50 mM KCl/2 mM dithiothreitol) and was incubated with various amounts of SELB for 10 min at 20°C in a volume of 10 μl. Samples were then placed at 4°C for 5 min and, after addition of 1 μl of 87% glycerol, applied to a 4% nondenaturing polyacrylamide gel (crosslink 60:1) in buffer K without KCl. Electrophoresis was performed at 4°C and 100 V for ≈3 h followed by autoradiography. Apart from the experiments described in Fig. 1*A*, all gel shift assays were performed in the presence of 0.5 mM GTP, which was also added to the gel matrix and electrophoresis buffer.

Footprinting Analysis. Formamide and sequencing reactions employing nucleases T1 (guanine specific, Boehringer Mannheim) and U2 (adenine specific, Boehringer Mannheim) were performed as described (28). Labeled wt transcript (1.5 pmol) was preincubated with SELB or storage buffer (buffer K in 50% glycerol) for 5 min at 20°C and then in buffer S (50 mM sodium cacodylate, pH 6.5/10 mM $MgCl_2$) for nuclease S1 reactions or buffer C (50 mM Tris·HCl, pH 7.2/10 mM $MgCl_2$) for cobra venom nuclease (CVN) reactions in a volume of 10 μl. Partial cleavage with 50 units of nuclease S1 (Pharmacia) and 0.025 units of CVN (Pharmacia) was performed for 5 min at 20°C followed by addition of 5 μl of 8 M urea/20% sucrose and dyes and freezing on dry ice. Samples were heated at 90°C for 1 min and subjected to electrophoresis on denaturing 12% polyacrylamide gels followed by autoradiography. Iodine cleavage footprints were carried out as described (26).

Synthesis of Selenocysteinyl-tRNA^{Sec}. Charging of tRNA^{Sec} with serine and conversion of seryl-tRNA^{Sec} to selenocysteinyl-tRNA^{Sec} were performed as described (29).

RESULTS

Interaction Between SELB and the Selenoprotein mRNA. The model proposed above implies that the targeted insertion of selenocysteine rests on the formation of a complex between SELB, selenocysteinyl-tRNA^{Sec}, and the mRNA recognition element in the presence of guanine nucleotides. An *in vitro* system was devised employing purified components to test this assumption. First, short mRNA transcripts were generated with the aid of phage T7 RNA polymerase that cover the *fdhF* mRNA segment containing the putative hairpin structure at the 3′ side of the UGA codon (see Fig. 3). An analogous transcript (termed C3) was also synthesized, which differs from the wt transcript in a single-base change (guanine versus cytosine) in the loop region of the secondary structure. This change has previously been shown to decrease drastically selenocysteine insertion directed by the UGA codon located 20 bases upstream (18).

Interaction of purified SELB protein with the wt transcript was then tested in a gel shift binding assay conducted in nondenaturing polyacrylamide gels. Stringent buffer conditions (100 mM potassium phosphate) had to be used to preclude nonspecific binding, since SELB is a "sticky" protein, which binds to column supports. Fig. 1*A* shows the effect of increasing amounts of SELB on transcript migration in gel shift assays in the absence of nucleotides and in the presence of ATP or GTP. A shift of the migration position of the transcript occurred in the absence of nucleotides or in the presence of ATP, but the bands were diffuse and a considerable amount of the complex was retained in the gel pockets when a large molar excess of SELB was employed. The solubility of the SELB·transcript complex, however, was clearly increased in the presence of guanine nucleotides, which can be taken as an indication (but not as proof) that the complex also contains bound GTP. This effect was specific for guanine nucleotides and not due to the alteration of the ionic strength since all experiments and the electrophoretic separation were performed in a high salt buffer. A similar effect of GTP on solubility of SELB had been noticed previously during purification of the protein (30). The migration of the SELB·transcript complex was somewhat faster when low concentrations of SELB were employed, a phenomenon that cannot be explained at present.

Next, it was tested whether the base change present in the C3 transcript affects the binding of SELB. Fig. 1*B* demonstrates that an increase in the amount of SELB, up to a 25-fold molar excess, leads to an essentially complete shift of the wt transcript but only to a partial shift of the C3 variant, even at very high SELB concentrations. It indicates that the drastic impairment of selenocysteine insertion conferred by the C3 mutation *in vivo* (18) is paralleled by a reduced affinity of the C3 transcript for translation factor SELB.

In competition experiments, unlabeled wt transcript effectively quenched complex formation between labeled wt transcript and SELB, whereas the C3 transcript, bulk tRNA, 5S rRNA, or uncharged tRNA^{Sec} did not (data not shown). Taken together, the results show that SELB undergoes a specific interaction with the wt transcript and that guanine nucleotides stabilize the complex formed.

Footprinting Analysis of the SELB·mRNA Interaction. It has been shown recently that decoding of the UGA in the *fdhF* mRNA with selenocysteine requires the presence of primary and secondary structure elements at the 3′ side of the codon (16, 18). Since base changes in the loop region of this secondary structure result in the elimination of or a decrease in selenocysteine insertion and, in the case of the C3 mutation, also in a reduced interaction of the transcript with SELB (see Fig. 1*B*), it can be assumed that the loop region may be

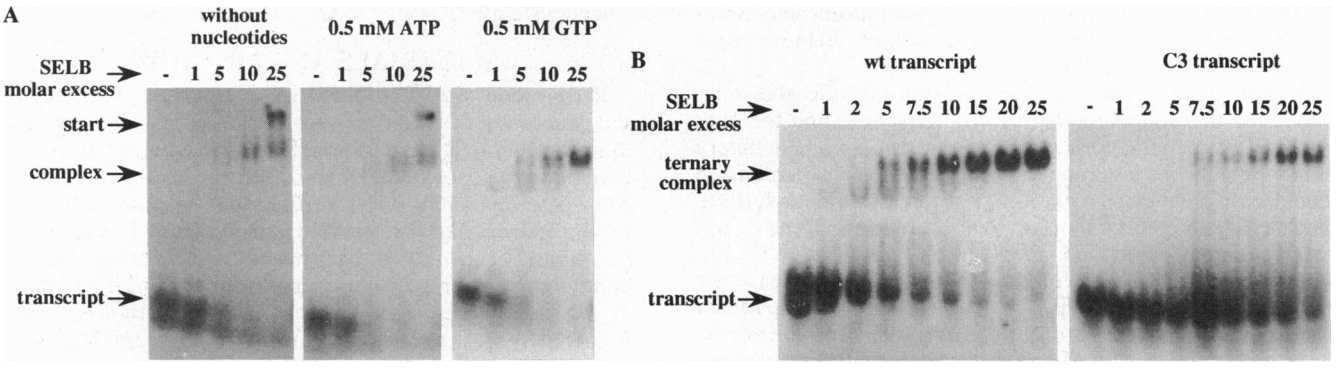

FIG. 1. Specificity of interaction between SELB and the wt transcript as tested in gel shift binding assays. (*A*) Nucleotide dependence. The wt transcript (1.5 pmol) and SELB, in the relative molar excess indicated, were incubated without nucleotides, with 0.5 mM ATP, or in the presence of 0.5 mM GTP and subjected to electrophoresis in gels containing the same concentration of nucleotides. SELB was preincubated with nucleotides for at least 3 h prior to its use in the assay. (*B*) Titration experiment comparing the binding of wt and C3 transcripts by SELB. Ternary complex, complex between SELB, mRNA, and possibly GTP.

part of the contact site with the protein. Different chemical and enzymic footprinting techniques were employed to test this assumption.

Single-strand-specific nuclease S1 cleaved the wt transcript at three positions in the loop region, and addition of SELB to the assay protected the mRNA from cleavage at these positions (Fig. 2*A*). Double-strand-specific CVN cleaved at several positions in the putative stem region of the transcript. Addition of SELB resulted in a weakening of some cuts in the 5′ stem region, whereas other cleavages in the 3′ stem were enhanced. These results support the notion that the loop of the hairpin structure is involved in binding the translation factor but does not prove it, since due to their size nucleases may be prevented from cleavage by a binding protein via steric hindrance from a more remote site. Therefore, to define the SELB binding site in more detail, we employed a footprinting technique based on cleavage of phosphorothioate-containing transcripts by iodine (26, 31). Phosphate ester bonds protected from iodine cleavage are believed to be in close contact with a binding protein because of the small size of this cleaving reagent. We found that SELB-dependent protection from iodine cleavage occurred at the tip of the loop region and that cleavage at a site nearby was enhanced (Fig. 2*B*).

Fig. 3 summarizes all the footprinting results. The differential susceptibility to cleavage by the S1 nuclease and CVN provides support for the de facto existence of the secondary structure in the transcript. Protection of the loop region from S1 and iodine cleavage support the notion that it is part of the SELB binding site. Binding of SELB may induce a conformational change of the hairpin structure as reflected by alterations of the CVN cleavage pattern. Addition of selenocysteinyl-tRNA^{Sec} did not change the cleavage or protection patterns with the probes we have employed (data not shown).

Formation of a Complex Between SELB, Selenocysteinyl-tRNA^{Sec}, and the mRNA in the Presence of GTP. It has previously been shown that translation factor SELB binds GTP and selenocysteinyl-tRNA^{Sec} but does not interact with the biosynthetic precursor seryl-tRNA^{Sec} (22). Therefore, it was of particular interest to determine whether binding of the mRNA and of selenocysteinyl-tRNA^{Sec} can take place concomitantly or whether they bind in a mutually exclusive manner. Gel shift assays were performed employing the wt transcript and SELB protein in the presence of increasing amounts of selenocysteinyl-tRNA^{Sec} or seryl-tRNA^{Sec}. In the experiments displayed in Fig. 4 *A* and *B*, the transcript was radioactively labeled; in those of Fig. 4 *C* and *D*, ^{14}C-labeled selenocysteinyl-tRNA^{Sec} and seryl-tRNA^{Sec}, respectively, were employed. Fig. 4*A* shows that addition of selenocysteinyl-tRNA^{Sec} to the gel shift assay resulted in a faster migration of the SELB·transcript complex, possibly due to the higher charge. Addition of seryl-tRNA^{Sec} (Fig. 4*B*)

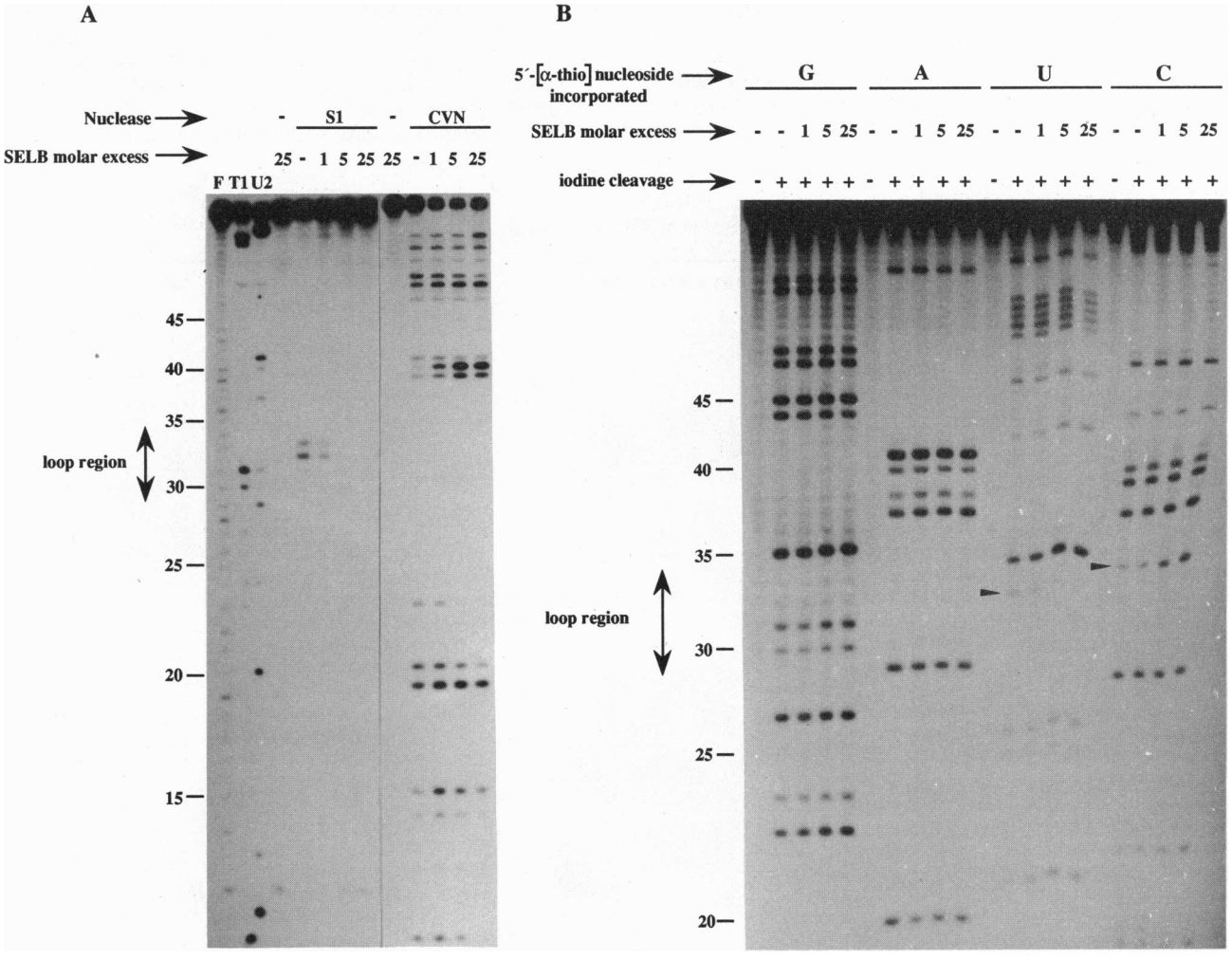

FIG. 2. Footprinting experiments of SELB–wt transcript interaction using different cleaving agents. (*A*) Transcripts were cleaved with nucleases S1 (single-strand-specific) or CVN (double-strand-specific) in the presence or in the absence of SELB. Formamide degradation (F) and sequencing reactions using nucleases T1 (guanine specific) and U2 (adenine specific) served to assign the positions of cleavage. (*B*) Transcripts substituted with [α-thio] nucleosides were subjected to iodine cleavage in the presence or in the absence of SELB. Enhanced or reduced cleavages in the presence of SELB are indicated by arrowheads.

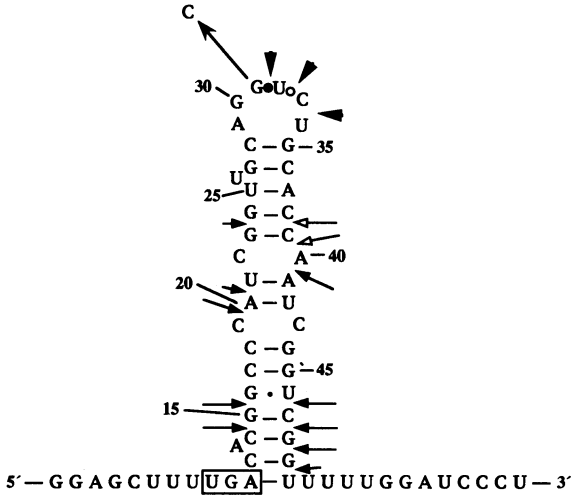

FIG. 3. Synopsis of the footprint results displayed on the secondary structure of the wt transcript. The long arrow indicates the base change in which the C3 transcript differs from the wt sequence. S1 cuts are denoted by arrowheads and CVN cuts are denoted by arrows. Positions protected from CVN cleavage, with unchanged reactivity, or enhanced reactivity after addition of SELB are denoted by short arrows, medium arrows, or medium arrows with open arrowheads, respectively. Protections from iodine cleavage or enhanced reactivities are denoted by closed and open circles, respectively.

did not cause any alteration of the migration behavior, which is in accord with the fact that SELB does not bind the biosynthetic precursor of selenocysteinyl-tRNA^{Sec} (22). When radioactively labeled selenocysteinyl-tRNA^{Sec} (Fig. 4*C*) and seryl-tRNA^{Sec} (Fig. 4*D*) and unlabeled transcript were used in the gel shift experiments, it was found that selenocysteinyl-tRNA^{Sec} was present in the SELB·transcript complex (Fig. 4*C*), which migrated to a faster position (Fig. 4*A*). Seryl-tRNA^{Sec}, on the other hand, did not enter any complex (Fig. 4*D*).

An intriguing feature of complex formation is that the yield of the SELB·mRNA complex is much higher in the presence of selenocysteinyl-tRNA^{Sec} than in its absence (Fig. 4*A*). A plausible explanation is that binding of the aminoacyl-tRNA induces a conformational change in SELB leading to an increased affinity for the mRNA. Other possibilities, however, cannot be excluded.

DISCUSSION

Based on the results described above, the model depicted in Fig. 5 is proposed for the function of SELB in the process of selenoprotein formation. It provides biochemical support for the mechanism of UGA decoding postulated recently on the basis of genetic experiments (18). SELB, the selenocysteine-specific elongation factor, forms a complex with selenocysteinyl-tRNA^{Sec} in the presence of GTP and binds to the recognition element at the 3′ side of the UGA codon. A ribosome approaching from 5′ partially melts the hairpin structure, and as the UGA codon reaches the ribosomal A site, selenocysteinyl-tRNA^{Sec} is delivered to the correct position for codon–anticodon interaction. In analogy to elongation factor EF-Tu, this process may be accompanied by GTP hydrolysis. After dissociation of tRNA^{Sec} from SELB, its affinity for the recognition element decreases and it can be displaced by the translating ribosome. An important question

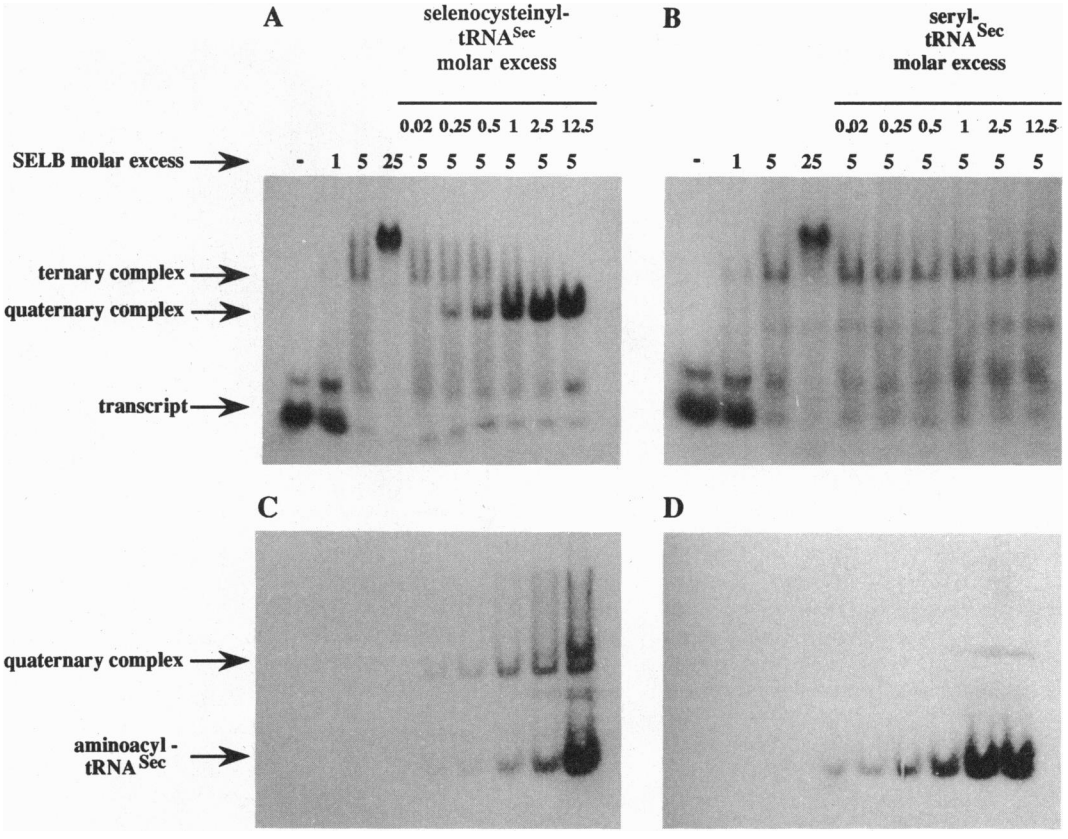

FIG. 4. Complex formation in the presence of charged tRNA^{Sec} as tested in gel shift binding assays. Increasing amounts of selenocysteinyl-tRNA^{Sec} or seryl-tRNA^{Sec} were incubated with SELB for 5 min at 20°C in buffer K, mixed with wt transcript, and subjected to electrophoresis in nondenaturing polyacrylamide gels. 5′-labeled wt transcript was used in *A* and *B*; ^{14}C-labeled selenocysteinyl-tRNA^{Sec} and seryl-tRNA^{Sec} were used in *C* and *D*. Ternary complex, complex between SELB, mRNA, and possibly GTP; quaternary complex, complex between SELB, mRNA, selenocysteinyl-tRNA^{Sec}, and possibly GTP.

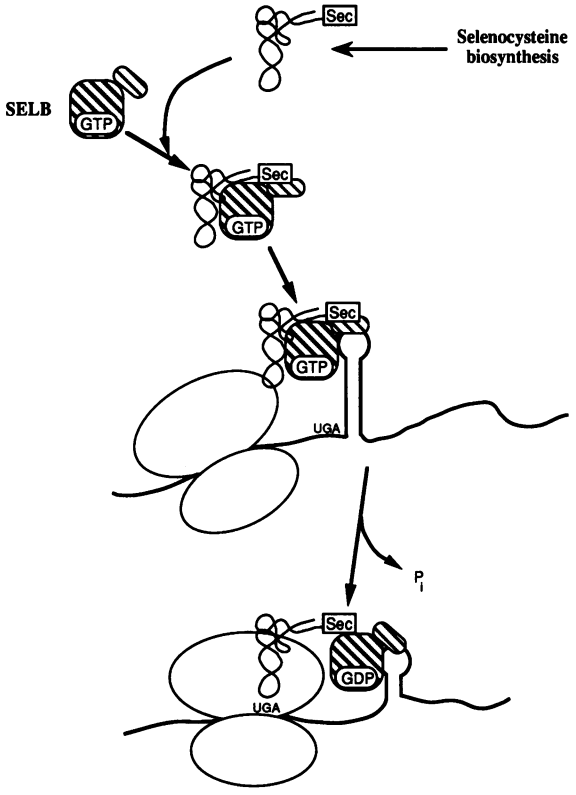

FIG. 5. Model for the cotranslational incorporation of selenocysteine (Sec) into *E. coli* proteins.

that still remains to be answered is which component of the system actually prevents termination. Possibilities are that either the hairpin structure itself or SELB bound to the recognition element may exert antitermination activity by hindering release factor 2 binding or activity.

Can the model elucidated here for the *E. coli* formate dehydrogenase H be applied as a general mechanism for selenocysteine insertion? The recognition elements within the mRNAs coding for different selenoproteins will certainly differ due to the sequence constraints of the particular enzyme. For example, insertion of selenocysteine into the large subunit of the [NiFeSe] hydrogenase of *Desulfomicrobium baculatum* (7) will necessitate another mRNA context compared with that of the *E. coli* formate dehydrogenase. If the model outlined above is a general one, it is to be expected, therefore, that SELB homologues exist in different organisms with different mRNA recognition properties. Attempts to express the glutathione peroxidase in *E. coli* failed (32), and a reason may reside in the incompatibility between the *gpx* mRNA and the *E. coli* SELB specificity.

What are the implications of this model for the eukaryotic system? Eukaryotic mRNA recognition elements (SECIS) identified so far are not localized close to the UGA codon but lie within the 3′ untranslated region of the selenoprotein mRNAs (19). It may well be that a eukaryotic homologue of SELB binds to the recognition element in the 3′ untranslated region of selenoprotein mRNAs and delivers selenocysteinyl-$tRNA^{Sec}$ to the ribosomal A site by looping out of the intervening mRNA region. As in *E. coli*, this model would lead to an increase in the local concentration of selenocysteinyl-$tRNA^{Sec}$ during the course of the translation of a selenoprotein mRNA. The development of a SECIS region that is interchangeable may have the advantage that within a eukaryotic cell a single SELB homologous translation factor could serve in the synthesis of different selenoproteins and also of selenoprotein P, which contains at least seven selenocysteine residues (15).

The investigation of selenoprotein synthesis in *E. coli* has thus led to the definition of an amino acid whose biosynthesis and insertion pathway are different from that of the 20 standard amino acids (4). SELB protein has proven to be particularly interesting because of its multiple functions as a guanine nucleotide-binding protein, selenocysteinyl-$tRNA^{Sec}$-specific elongation factor, and mRNA-binding protein that confers selenocysteine identity to specialized UGA codons. The fact that the yield or stability of the SELB·mRNA complex is drastically increased in the presence of selenocysteinyl-$tRNA^{Sec}$ is a feature that may contribute to the mechanism proposed.

We thank A. Herzog for the donation of purified SELB and P. Tormay for the gift of purified selenocysteine synthase and SELD protein. We are indebted to R. Giegé (Institut de Biologie Moléculaire et Cellulaire du CNRS, Strasbourg, France) and his group for advice in learning RNA footprinting methods. C.B. was supported by an EMBO short-term fellowship during his stay in Strasbourg. This work was supported by the Bundesministerium für Forschung und Technologie and the Fonds der Chemischen Industrie.

1. Atkins, J. F., Weiss, R. B. & Gesteland, R. F. (1990) *Cell* **62,** 413–423.
2. Gesteland, R. F., Weiss, R. B. & Atkins, J. F. (1992) *Science* **257,** 1640–1641.
3. Stadtman, T. C. (1990) *Annu. Rev. Biochem.* **59,** 111–127.
4. Böck, A., Forchhammer, K., Heider, J. & Baron, C. (1991) *Trends Biochem. Sci.* **16,** 463–467.
5. Zinoni, F., Birkmann, A., Stadtman, T. C. & Böck, A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4650–4654.
6. Chambers, I., Frampton, J., Goldfarb, P., Affara, N., McBain, W. & Harrison, P. R. (1986) *EMBO J.* **5,** 1221–1227.
7. Menon, N. K., Peck, H. D., Jr., LeGall, J. & Przybyla, A. E. (1988) *J. Bacteriol.* **170,** 4429.
8. Heider, J., Forchhammer, K., Sawers, G. & Böck, A. (1991) *Arch. Microbiol.* **155,** 221–228.
9. Garcia, G. E. & Stadtman, T. C. (1991) *J. Bacteriol.* **173,** 2093–2098.
10. Berg, B. L., Li, J., Heider, J. & Stewart, V. (1991) *J. Biol. Chem.* **266,** 22380–22385.
11. Halboth, S. & Klein, A. (1992) *Mol. Gen. Genet.* **233,** 217–224.
12. Takahashi, K., Akasaka, M., Yamamoto, Y., Kobayashi, C., Mizoguchi, J. & Koyama, J. (1990) *J. Biochem.* **108,** 145–148.
13. Schuckelt, R., Brigelius-Flohé, R., Maiorino, M., Roveri, A., Reumkens, J., Strassburger, W., Ursini, F., Wolf, B. & Flohé, L. (1991) *Free Rad. Res. Comms.* **14,** 343–361.
14. Berry, M. L., Banu, L. & Larson, P. R. (1991) *Nature (London)* **349,** 438–440.
15. Hill, K. E., Lloyd, R. S., Yang, J. G., Read, R. & Burk, R. F. (1991) *J. Biol. Chem.* **266,** 10050–10053.
16. Zinoni, F., Heider, J. & Böck, A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4660–4664.
17. Berg, B. L., Baron, C. & Stewart, V. (1991) *J. Biol. Chem.* **266,** 22386–22391.
18. Heider, J., Baron, C. & Böck, A. (1992) *EMBO J.* **11,** 3759–3766.
19. Berry, M. J., Banu, L., Chen, Y., Mandel, S. J., Kieffer, J. D., Harney, J. W. & Larson, P. R. (1991) *Nature (London)* **353,** 273–276.
20. Baron, C., Heider, J. & Böck, A. (1990) *Nucleic Acids Res.* **18,** 6761–6766.
21. Leinfelder, W., Zehelein, E., Mandrand-Berthelot, M. A. & Böck, A. (1988) *Nature (London)* **331,** 723–725.
22. Forchhammer, K., Leinfelder, W. & Böck, A. (1989) *Nature (London)* **342,** 453–456.
23. Forchhammer, K., Rücknagel, K. P. & Böck, A. (1990) *J. Biol. Chem.* **265,** 9346–9350.
24. Rosenberg, A. H., Lade, B. N., Chui, D. S., Lin, S. W., Dunn, J. J. & Studier, F. W. (1987) *Gene* **56,** 125–135.
25. Wyatt, J. R., Chastain, M. & Puglisi, J. D. (1991) *BioTechniques* **11,** 764–769.
26. Schatz, D., Leberman, R. & Eckstein, F. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 6132–6136.
27. Silberklang, M., Gillam, A. M. & RajBhandary, U. L. (1977) *Nucleic Acids Res.* **4,** 4091–4108.
28. Donis-Keller, H., Maxam, A. M. & Gilbert, W. (1977) *Nucleic Acids Res.* **4,** 2527–2538.
29. Baron, C. & Böck, A. (1991) *J. Biol. Chem.* **266,** 20375–20379.
30. Forchhammer, K. (1991) Ph.D. thesis (University of Munich, F.R.G.).
31. Rudinger, J., Puglisi, J. D., Pütz, J., Schatz, D., Eckstein, F., Florentz, C. & Giegé, R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5882–5886.
32. Rocher, C., Faucheu, C., Hervé, F., Bénicourt, C. & Lalanne, J. L. (1991) *Gene* **98,** 193–200.