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Interaction of the *Escherichia coli fdhF* mRNA hairpin promoting selenocysteine incorporation with the ribosome

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ABSTRACT

The codon UGA located 5′ adjacent to an mRNA hairpin within *fdhF* mRNA promotes the incorporation of the amino acid selenocysteine into formate dehydrogenase H of *Escherichia coli*. The loop region of this mRNA hairpin has been shown to bind to the special elongation factor SELB, which also forms a complex with selenocysteinyl-tRNA^{Sec} and GTP. We designed seven different mRNA constructs derived from the *fdhF* mRNA which contain a translation initiation region including an AUG initiation codon followed by no, one, two, three, four, five or six UUC phenylalanine codon(s) and the UGA selenocysteine codon 5′ adjacent to the *fdhF* mRNA hairpin. By binding these different mRNA constructs to 30S ribosomal subunits *in vitro* we attempted to mimic intermediate steps of elongation of a structured mRNA approaching the ribosome by one codon at a time. Toeprint analysis of the mRNA–ribosome complexes showed that the presence of the *fdhF* mRNA hairpin strongly interferes with binding of the *fdhF* mRNA to 30S ribosomal subunits as soon as the hairpin is placed closer than 16 bases to the ribosomal P-site. Binding is reduced up to 25-fold compared with mRNA constructs where the hairpin is located outside the ribosomal mRNA track. Surprisingly, no toeprint signals were observed in any of our mRNA constructs when tRNA^{Sec} was used instead of tRNA^{fMet}. Lack of binding of selenocysteinyl-tRNA^{Sec} to the UGA codon was attributed to steric hindrance by the *fdhF* mRNA hairpin. By chemical probing of the shortest mRNA construct (AUG-UGA-fdhF hairpin) bound to 30S ribosomal subunits we demonstrate that the hairpin structure is not unfolded in the presence of ribosomes *in vitro*; also, this mRNA is not translated *in vivo* when fused in-frame 5′ of the *lacZ* gene. Therefore, our data indicate that the *fdhF* mRNA hairpin has to be unfolded during elongation prior to entering the ribosomal mRNA track and we propose that the SELB binding domain within the *fdhF* mRNA is located outside the ribosomal mRNA track during decoding of the UGA selenocysteine codon by the SELB–selenocysteinyl-tRNA^{Sec}–GTP complex.

INTRODUCTION

There is ample evidence that specific mRNA secondary structures are required for so-called recoding events during translation of certain mRNAs (1,2). However, very little is known about the direct interaction of these mRNA secondary structures with the ribosome (3–5). Several questions as to when and where these mRNA structures interact with the ribosome remain unanswered so far and it is still unclear when and by which mechanism(s) these mRNA structures become unfolded during translation (6). While the interaction of structured mRNAs with the ribosome during translation initiation in *Escherichia coli* has been intensively studied (for reviews see 7–10) their interaction with the ribosome during elongation is obscured by the dynamic process of this event.

In this respect, our study represents an initial approach to elucidate the interaction of an mRNA secondary structure with the ribosome during elongation. We chose the RNA hairpin within *fdhF* mRNA, which promotes the incorporation of selenocysteine into formate dehydrogenase H in *E.coli*. This hairpin, located 3′ adjacent to the UGA selenocysteine codon (11,12), is a prerequisite for selenocysteine incorporation (13). Besides its presence, the incorporation pathway requires a specific tRNA (selenocysteinyl-tRNA^{Sec}) containing a UCA anticodon complementary to the UGA codon as well as a specific elongation factor, designated SELB. Elongation factor SELB, exhibiting extensive sequence similarity to EF-Tu (14), was shown to bind to selenocysteinyl-tRNA^{Sec} (14) as well as to the loop region of the mRNA hairpin (12,15). It is assumed that by this mechanism selenocysteinyl-tRNA^{Sec} is tethered to the UGA selenocysteine codon.

We performed toeprinting assays of mRNA constructs containing one, two, three, four, five or six codons between an AUG initiation codon (preceded by a Shine–Dalgarno sequence) and the UGA selenocysteine codon adjacent to the mRNA hairpin. By using these constructs we attempted to mimic intermediate steps of elongation of the *fdhF* mRNA on the ribosome by moving the hairpin closer to the decoding site by one codon at a time. The objective of this study was to gain a first insight into the interaction of the *fdhF* mRNA stem–loop structure with the translation apparatus.

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MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma (Germany) unless indicated otherwise. T7 polymerase was a generous gift from Thomas Maier (München), AMV reverse transcriptase was purchased from Appligene (France) and kethoxal was supplied by Upjohn (UK).

tRNAs

$tRNA^{Phe}$ and $tRNA^{fMet}$ were obtained from Sigma (Germany) and $tRNA^{Sec}$ was a generous gift from Christian Baron (München).

mRNAs

mRNAs were transcribed from DNA templates containing a T7 promotor (16) followed by a translational initiation region including an AUG initiation codon and the UGA selenocysteine codon 5′ adjacent to the *fdhF* hairpin. Spacing between the AUG and UGA codon was designed containing no (AH75 $[UUC]_0$), one (AH78 $[UUC]_1$), two (AH81 $[UUC]_2$), three (AH84 $[UUC]_3$), four (AH87 $[UUC]_4$), five (AH90 $[UUC]_5$) or six (AH93 $[UCC]_6$) UUC phenylalanine codons. DNA templates were generated by PCR amplification of plasmid DNA pAF1 (12) essentially as described by Saiki *et al.* (17), using the following primers.

5′-Primers
AH75, 5′-GGC ACA TGT TAA TAC GAC TCA CTA TAG GGC TAA ATT TTG GAG GCA TTA **ATG TGA** CAC GGC CCA TCG GTT GCA GGT-3′;
AH78, 5′-GGC ACA TGT TAA TAC GAC TCA CTA TAG GGC TAA ATT TTG GAG GCA TTA **ATG** TTC **TGA** CAC GGC CCA TCG GTT GCA GGT-3′;
AH81, 5′-GGC ACA TGT TAA TAC GAC TCA CTA TAG GGC TAA ATT TTG GAG GCA TTA **ATG** TTC TTC **TGA** CAC GGC CCA TCG GTT GCA GGT-3′;
AH84, 5′-GGC ACA TGT TAA TAC GAC TCA CTA TAG GGC TAA ATT TTG GAG GCA TTA **ATG** TTC TTC TTC **TGA** CAC GGC CCA TCG GTT GCA GGT-3′;
AH87, 5′-GGC ACA TGT TAA TAC GAC TCA CTA TAG GGC TAA ATT TTG GAG GCA TTA **ATG** TTC TTC TTC TTC **TGA** CAC GGC CCA TCG GTT GCA GGT-3′;
AH90, 5′-GGC ACA TGT TAA TAC GAC TCA CTA TAG GGC TAA ATT TTG GAG GCA TTA **ATG** TTC TTC TTC TTC TTC **TGA** CAC GGC CCA TCG GTT GCA GGT-3′;
AH93, 5′-GGC ACA TGT TAA TAC GAC TCA CTA TAG GGC TAA ATT TTG GAG GCA TTA **ATG** TTC TTC TTC TTC TTC TTC **TGA** CAC GGC CCA TCG GTT GCA GGT-3′.

ATG and TGA sequences are indicated in bold. 5′-Primers contained an *Afl*III site, the T7 promotor sequence, the translation initiation region including the AUG codon as well as no (AH75), one (AH78), two (AH81), three (AH84), four (AH87), five (AH90) or six (AH93) TTC phenylalanine codons followed by a region complementary to the *fdhF* mRNA hairpin (positions 418–441); numbering according to Zinoni *et al.* (18)

3′-Primer
AM2/T, 5′-GGC GGA TCC TCG GTA TTA TCA ATT TCG TTA ATA GC-3′.

The 3′-primer contained a *Bam*HI site followed by a sequence complementary to a region downstream of the *fdhF* hairpin (positions 478–503); numbering according to Zinoni *et al.* (18). Purification of DNA oligonucleotides and T7 transcription were performed as described by Hüttenhofer and Noller (5).

Preparation of ribosomes and ribosomal subunits

Escherichia coli 600 MRE 0.5 M salt washed 70S ribosomes were prepared as described by Moazed and Noller (19) and 30S subunits were obtained as described by Moazed *et al.* (20). 30S subunits were activated by heating in reaction buffer A (10 mM $MgCl_2$, 140 mM NH_4Cl, 80 mM potassium cacodylate, pH 7.2) at 42°C for 20 min before being used for mRNA and tRNA binding (21).

Binding of mRNAs to ribosomes and toeprint assays

Binding of mRNAs to 30S ribosomal subunits was performed by incubating 1 pmol mRNA, 7 (or 14) pmol 30S ribosomal subunits and 40 pmol tRNA in the absence or presence of 45 pmol SELB in reaction buffer B (10 mM Tris–acetate, pH 7.4, 60 mM NH_4Cl, 10 mM Mg-acetate, 0.5 mM GTP, 2 mM DTT). The ^{32}P-end-labeled AM2/T primer (see above), which is complementary to the 3′-end of the various RNA transcripts, was annealed as described in Hartz *et al.* (22). Toeprinting assays were performed according to Ringquist *et al.* (23). Quantification of toeprints was performed on a Molecular Dynamics Personal Densitometer. Binding affinity of mRNAs was assessed as the intensity of the +16 toeprint signal divided by the sum of the full-length signal plus the +16 toeprint (%).

Chemical probing of the AH75 $[UUC]_0$–30S complex with kethoxal (KE)

Chemical probing of mRNA–ribosome complexes with KE was performed as described by Hüttenhofer and Noller (5). Primer extension reactions for analysis of modified bases were performed as described by Stern *et al.* (24), using 5′-^{32}P-end-labeled primer AM2/T (see above). Samples were loaded onto 6% (w/v) polyacrylamide–7 M urea gels. Electrophoresis was performed at 2000 V, 22 mA for 1.5 h.

Cloning of the the AUG-UGA mRNA construct and expression *in vivo*

A translational fusion was constructed which consisted of a translational initiation codon directly fused to a UGA codon followed by the *fdhF* hairpin and the *lacZ* gene. To this end, we amplified a 140 bp PCR product from plasmid pWT which contains a translational fusion of a selenium insertion cartridge into the *lacZ* gene (11). Amplification was achieved with a 5′-primer containing the desired mutations and a 3′-primer complementary to a region of the *lacZ* gene (positions 6291–6306).

5′-primer, 5′-GGA AGC TTA AGG AGG AAA TTA TTA TGT GAC ACG GCC CAT GC-3′;
3′-primer, 5′-GTA AAA CGA CGG CCA GT-3′.

The amplification product was cloned into plasmid pSKS106 utilising *Hin*dIII and *Bam*HI sites introduced at the insert borders (25). The *Hin*dIII site was filled in with the Klenow enzyme. The resulting plasmid contained the *lac* promotor followed by an artificial reading frame of seven codons that was terminated by a UAG codon and the AUG-UGA-*fdhF* hairpin–*lacZ* gene fusion preceded by an optimised Shine–Dalgarno sequence. Selenocysteine insertion into fusion proteins was assessed by measuring β-galactosidase activity obtained with this construct and plasmid

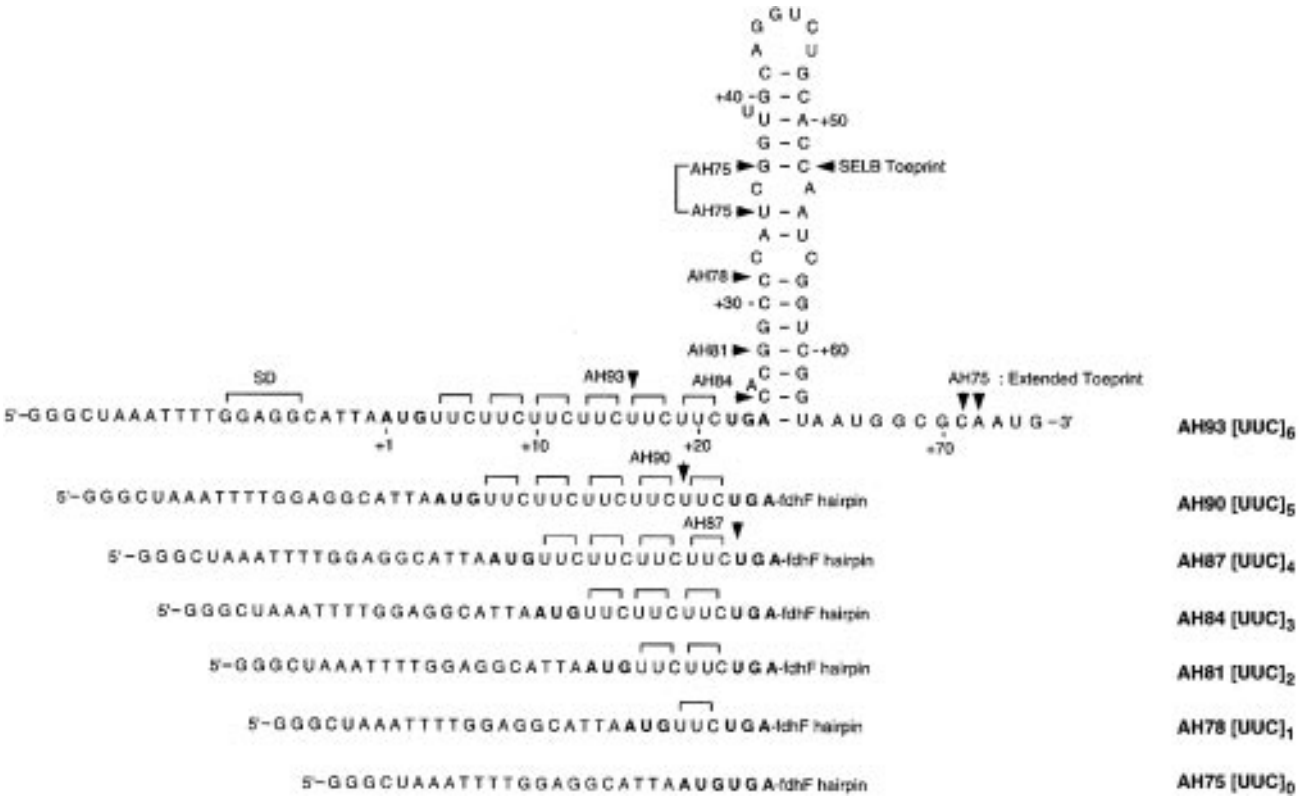

Figure 1. Sequence of mRNA constructs (AH93 $[UUC]_6$–AH75 $[UUC]_0$) used for toeprint analysis. The position of the toeprint signals (+16 toeprints) due to binding of mRNAs to 30S ribosomal subunits as well as the toeprint induced by special elongation factor SELB (position +52) are indicated by arrow heads. The AH75 $[UUC]_0$ mRNA construct results in three ribosome-dependent toeprint signals at positions +16, +18 and +71/72 ('extended toeprint').

pWT (11). *Escherichia coli* strains FM434 and FM464 (13) were transformed with the plasmids and analysed for synthesis of β-galactosidase as previously described (13).

RESULTS

Toeprint analysis of the interaction of AH75 $[UUC]_0$–AH90 $[UCC]_5$ mRNAs with 30S ribosomal subunits

We performed toeprinting studies of ribosome–mRNA complexes using mRNA constructs containing no, one, two, three, four or five UUC phenylalanine codon(s) between the UGA selenocysteine codon adjacent to the *fdhF* hairpin and the AUG initiation codon (AH75 $[UUC]_0$–AH90 $[UUC]_5$, Figs 1 and 2). The mRNA constructs were bound to 30S ribosomal subunits in the presence of equal amounts of $tRNA^{fMet}$ (see Materials and Methods) and toeprints assays were performed in the presence or absence of special elongation factor SELB. Elongation factor SELB has been shown previously to bind to the loop region of the *fdhF* hairpin (12,15). In the absence of SELB the intensities of the +16 toeprint signals strongly decrease when the *fdhF* hairpin approaches the ribosomal decoding site; this is achieved by reducing the distance between the AUG and UGA codon gradually by one codon at a time. Reduction in intensities of toeprint signals indicates a reduced binding of these mRNA constructs to the 30S ribosomal subunits. Thereby, binding was assessed as the ratio of the +16 toeprint with respect to the full-length cDNA (see Materials and Methods). Densitometric evaluation of toeprint signals showed that 45% of AH90 $[UUC]_5$ mRNA bound to 30S ribosomal subunits and an up to 25-fold reduction in binding of the remaining mRNA constructs (Figs 2 and 3). About 47% of an mRNA construct containing a spacer region of six codons, AH93 $[UUC]_6$, was bound to 30S ribosomal subunits, comparable with binding of the AH90 $[UUC]_5$ mRNA (Fig. 1 and data not shown).

Surprisingly, an additional ~3-fold stronger toeprint signal was observed when the AH75 $[UUC]_0$ mRNA (the AUG-UGA construct) was bound to 30S ribosomal subunits (Fig. 2). This toeprint corresponds to +18 bases downstream from A+1. The most likely explanation for the +18 toeprint is that $tRNA^{fMet}$ binds to the AH75 $[UUC]_0$ mRNA within two different reading frames; the +16 toeprint being due to decoding of $tRNA^{fMet}$ at AUG-UGA, the +18 toeprint due to decoding of $tRNA^{fMet}$ at AUG-UGA (e.g. two bases downstream of the first position). In addition, an 'extended' toeprint signal was observed within AH75 $[UUC]_0$ mRNA at positions +71/72, in agreement with data reported by Rinquist *et al.* (23). We fail, however, to detect the extended toeprint signal with all other constructs; this extended signal should be shifted by three bases at a time within the AH78 $[UUC]_1$–AH90 $[UUC]_5$ mRNAs as the spacing between the AUG and UGA codon in these mRNAs is gradually increased by one codon each (Figs 1 and 2).

A SELB-dependent toeprint signal is observed at position +52 (Figs 1 and 2), in agreement with previous data (23). This toeprint signal was shown not to be due to the presence of 30S ribosomal subunits, but to SELB binding to the loop region of the *fdhF* mRNA structure (23). Binding of SELB to the mRNA results in

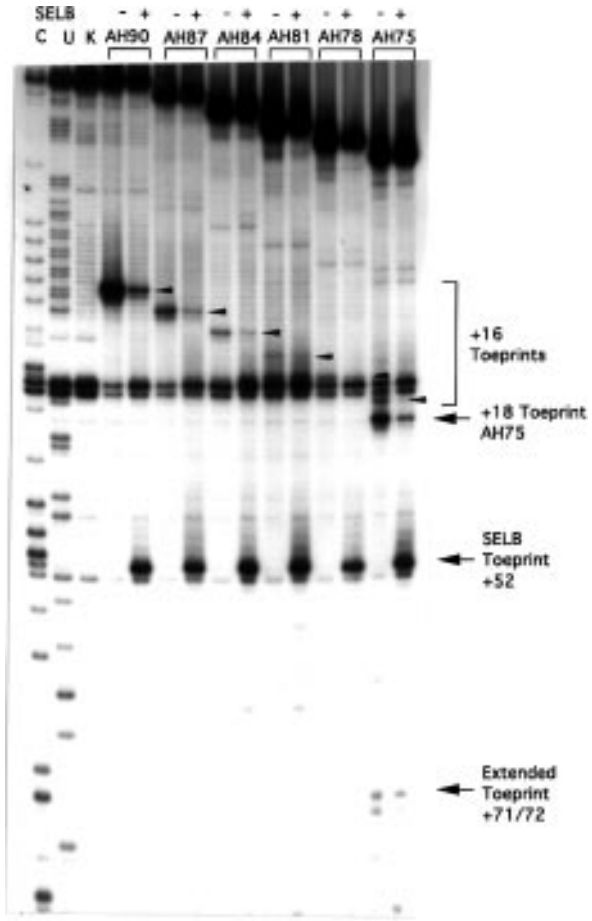

Figure 2. Autoradiograph of toeprinting experiments of mRNAs AH75 $[UUC]_0$–AH90 $[UUC]_5$ bound to 30S ribosomal subunits. C and U, sequencing lanes; K, control lane, no 30S ribosomal subunits added; all other lanes, +30S ribosomal subunits (see Materials and Methods). The addition of special elongation factor SELB is indicated. Relative positions of the +16 toeprint (AH75 $[UUC]_0$–AH90 $[UUC]_5$), +18 toeprint (AH75 $[UUC]_0$ only), SELB toeprint (position +52) and extended toeprint (+71/72) are indicated by arrows. Note the presence of a double band in all lanes due to stalling of reverse transcriptase by the the *fdhF* hairpin structure.

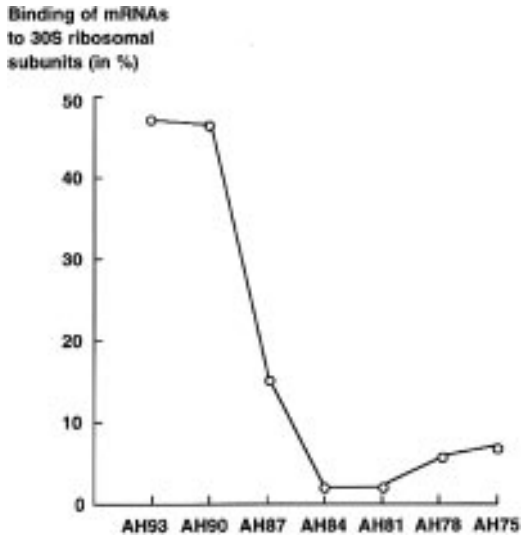

Figure 3. Quantification of binding of AH75 $[UUC]_0$–AH93 $[UUC]_6$ mRNAs to 30S ribosomal subunits (%) as assessed by toeprint analysis (+16 toeprint). Binding was determined by densitometric evaluation of +16 toeprint signals compared with full-length cDNAs (see Materials and Methods).

stalling of AMV reverse transcriptase at position +52; consequently, the intensity of the +16 toeprint signal decreases within every construct. However, the relative position of the +16 toeprint remains unaffected in the presence of SELB within all constructs used, indicative of SELB not positioning the mRNAs with respect to the ribosome (Fig. 2).

Influence of $tRNA^{fMet}$, $tRNA^{Phe}$ and $tRNA^{Sec}$ on the extended toeprint signal in AH75 $[UUC]_0$ and AH78 $[UUC]_1$ mRNAs

Next, we wanted to determine whether the extended toeprint signal was solely due to the presence of $tRNA^{fMet}$ positioning the mRNA with respect to the ribosome. To enhance the intensity of the toeprint signals a 14-fold excess of ribosomes over mRNAs was used, instead of the 7-fold excess used for the previous experiment (see Materials and Methods). We incubated the AH78 $[UUC]_1$ mRNA (the AUG-UUC-UGA construct) with 30S ribosomal subunits in the presence of $tRNA^{fMet}$, $tRNA^{Phe}$ or $tRNA^{Sec}$ and compared the resulting toeprint signals to those obtained with the AH75 $[UUC]_0$ mRNA (the AUG-UGA construct) in the presence of $tRNA^{fMet}$ and $tRNA^{Sec}$. Figure 4 shows binding of the AUG-UUC-UGA mRNA to 30S ribosomal subunits in the presence of $tRNA^{fMet}$, which results in a toeprint at position +16, however, no extended toeprint is visible. Binding of $tRNA^{Phe}$ to the UUC codon results in a toeprint shifted by three bases, as expected. In addition, an extended toeprint is visible at position +71/72 (Fig. 4), which is also observed within the AH75 $[UUC]_0$ mRNA (the AUG-UGA construct) when $tRNA^{fMet}$ is bound to the ribosome; note again the presence of two toeprint signals at positions +16 and +18 due to binding of $tRNA^{fMet}$ to AUG or GUG. Surprisingly, with $tRNA^{Sec}$ as an initiator tRNA neither the +16 nor extended toeprint is visible when the AH78 $[UUC]_1$ or AH75 $[UUC]_0$ mRNAs are bound to the ribosome, despite the fact that the UGA selenocysteine codon is located within a proper toeprint distance from the Shine–Dalgarno sequence.

At position 72, in the presence of any tRNA (fMet, Phe or Sec) and with both constructs used, there is a weak background toeprint signal observed in some of our experiments; the position of the signal is not shifted by three bases, as is the case for the +16 toeprints in the presence of the different tRNAs. We therefore attribute this signal, as well as the extended toeprint signal, to a conformational change within the mRNA upon binding to 30S ribosomal subunits (see Discussion).

Footprint analysis of the AH75 $[UUC]_0$ mRNA–30S ribosomal subunit complex

One of the difficulties in interpreting toeprint signals is their lack of information on the structural changes an mRNA might undergo upon binding to the ribosome. We therefore performed a footprint analysis of the AH75 $[UUC]_0$ mRNA–ribosome complex to investigate whether the mRNA secondary structure becomes unfolded upon binding to 30S ribosomal subunits. As a chemical probe to investigate the accessiblity of G bases, KE was used.

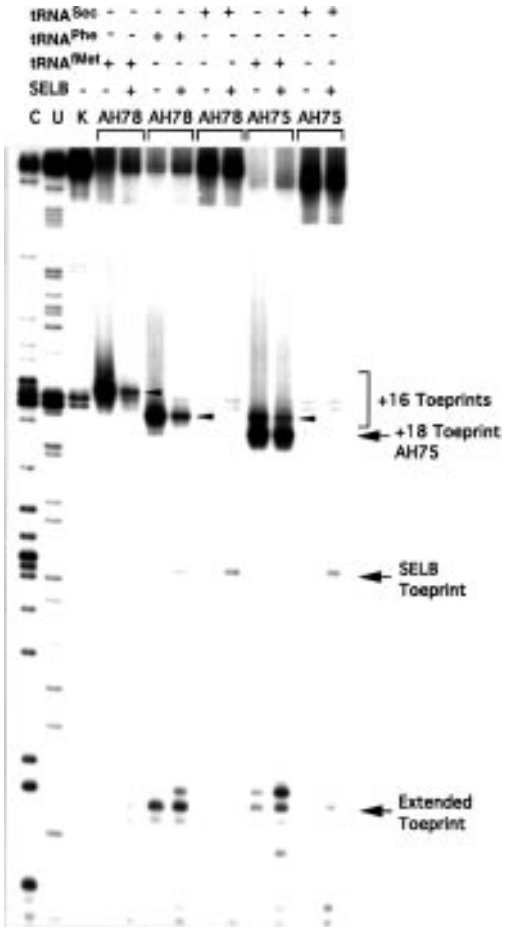

Figure 4. Autoradiograph of toeprint experiments of AH78 $[UUC]_1$ or AH75 $[UUC]_0$ mRNAs bound to 30S ribosomal subunits and SELB in the presence of $tRNA^{fMet}$, $tRNA^{Phe}$ or $tRNA^{Sec}$, as indicated. C and U, sequencing lanes; K, control lane, no 30S ribosomal subunits added; all other lanes, +30S ribosomal subunits (see Materials and Methods). Relative positions of the +16 toeprint, +18 toeprint (AH75 $[UUC]_0$ only), SELB and extended toeprints are indicated by arrows.

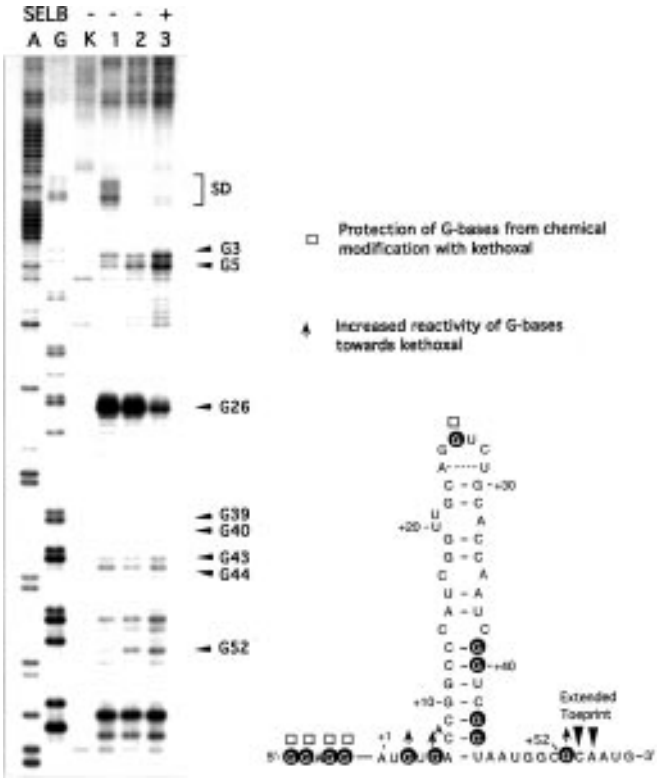

Figure 5. Autoradiograph of footprint analysis of the AH75 $[UUC]_0$ mRNA–30S ribosomal subunit complex. The AH75 $[UUC]_0$ mRNA was probed with KE in the presence or absence of 30S ribosomal subunits/$tRNA^{fMet}$ and special elongation factor SELB. Modified bases were detected by primer extension analysis (see Materials and Methods). A and G, sequencing lanes; K, control lane, no KE added; lane 1, AH75 $[UUC]_0$ mRNA alone; lane 2, + 30S ribosomal subunits and $tRNA^{fMet}$; lane 3, + 30S ribosomal subunits, $tRNA^{fMet}$ and elongation factor SELB. Protections or enhanced reactivities of G bases towards KE due to interaction with 30S ribosomal subunits or SELB are indicated; the position of the extended toeprint within AH75 $[UUC]_0$ mRNA (Fig. 2) is also shown. SD, Shine–Dalgarno sequence GGAGG.

Modified bases were analysed by primer extension analysis (see Materials and Methods). Unfolding of the stem–loop structure by the ribosome can be monitored by the increased accessibility of G bases in the stem structure of the hairpin. By chemical probing these G bases have been shown not to be accessible (G39, G40 and G43) or weakly accessible (G44) to chemical modification by KE in the free mRNA (12; Fig. 5, lane 1). Binding of the mRNA was performed in the presence or absence of special elongation factor SELB.

Quantitative binding of the AH75 $[UUC]_0$ mRNA is demonstrated by the complete protection of G bases of the Shine–Dalgarno sequence from chemical modification in the presence of 30S ribosomal subunits (Fig. 5, lane 2). However, no increased accessibilty of G bases in the stem structure of the RNA hairpin can be observed, indicating that no unfolding of the mRNA hairpin occurs (Fig. 5). In the presence of SELB (Fig. 5, lane 3), G+26 in the loop of the mRNA hairpin becomes protected from modification by KE due to interaction with SELB, as shown previously (12). Surprisingly, despite the presence of $tRNA^{fMet}$, G+3 of the AUG initiation codon is not protected from chemical modification. Instead, a slight increase in the reactivity of G+3 and G+5 towards KE is observed in the presence of SELB. A moderate increase in reactivitiy towards KE is also visible for base G+52 (Fig. 5, lanes 2 and 3; note the different numbering of bases compared with Fig. 1).

In vivo expression of the AUG-UGA–*lacZ* mRNA

To investigate *in vivo* expression of the AH75 $[UUC]_0$ mRNA (the AUG-UGA mRNA construct), we cloned the *fdhF* hairpin sequence, including the Shine–Dalgarno sequence and ATG initiation codon, in-frame 5′ of the *lacZ* gene (see Materials and Methods) and measured β-galactosidase synthesis of the resulting plasmid in bacterial strains FM434 and FM464 (13); spacing between the Shine–Dalgarno sequence and the ATG start codon was optimized to be 7 instead of 5 nt, to avoid out-of-frame decoding by $tRNA^{fMet}$ at the GUG sequence (see Discussion). Expression of β-galactosidase was compared with a construct, pATG-$(NNN)_7$-TGA, where a spacer region of seven codons was introduced between the ATG and TGA codons (11). As can be seen in Table 1, reduction of the distance between the ATG and TGA codons from seven codons to none resulted in a dramatic

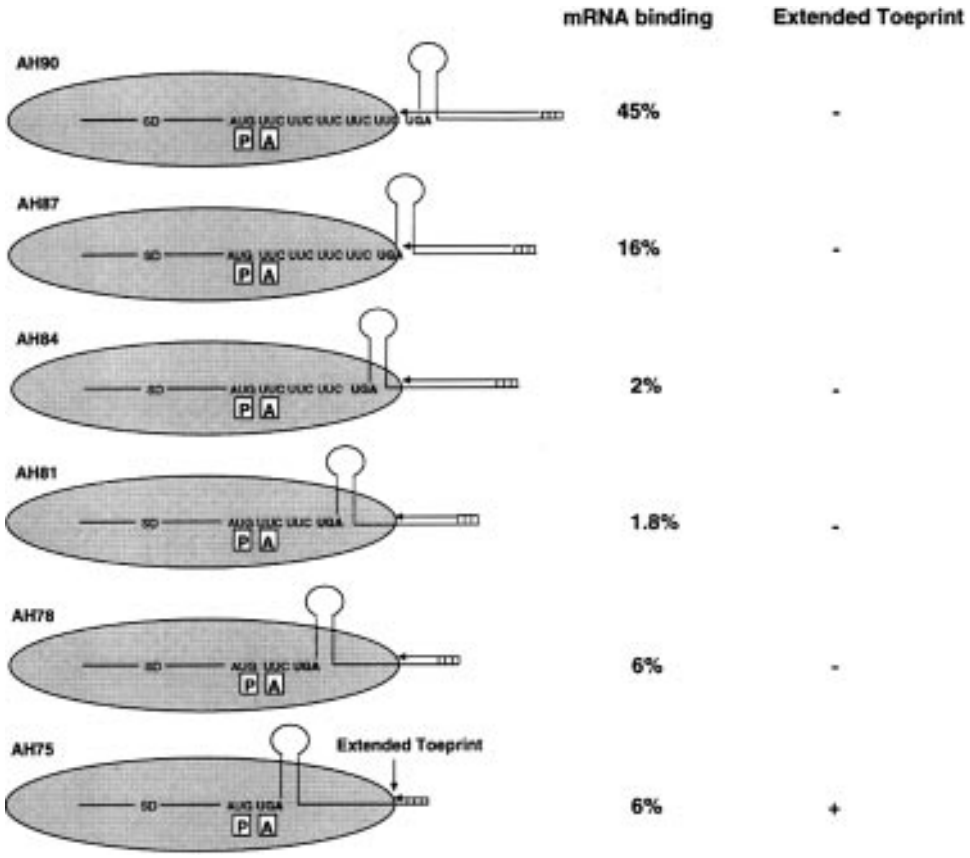

Figure 6. Toeprint scheme of the mRNA–30S ribosomal subunit complexes used in this study. The relative position of the *fdhF* hairpin structure with respect to the ribosome is indicated assuming the 'entry site' of the ribosomal mRNA track to be located between 16 and 19 bases downstream of the first base of the ribosomal P-site codon (5,26). Binding of the respective mRNA constructs to 30S ribosomal subunits as assessed by toeprint analysis shown (%) (see Material and Methods). The positions of the expected extended toeprint signals are indicated by arrows. The experimentally observed extended toeprint signal at position +52 within AH75 $[UUC]_0$ mRNA is shown.

decrease in β-galactosidase synthesis. Expression of the AUG-UGA construct in strain FM464 (13), lacking selenocysteine-tRNA (by deletion of the $tRNA^{Sec}$, encoding *selC* gene), resulted in the same decrease in β-galactosidase synthesis (Table 1). This indicates that UGA read-through and β-galactosidase expression is reduced to background levels in the AUG-UGA mRNA construct.

Table 1. Readthrough analysis with *fdhF-lacZ* gene fusions containing no [pATG-TGA] or seven codons [pATG-$(NNN)_7$-TGA] between the AUG start and UGA stop codon

Strain	Plasmid	Miller units
FM434	pATG-TGA	6
FM464 (ΔselC)	pATG-TGA	8
FM434	pATG-$(NNN)_7$-TGA	1450

Bacterial strain FM434, or FM464 which lacks the $tRNA^{Sec}$ gene (ΔselC), were used as hosts for transformation of plasmids.

DISCUSSION

In this study we show that the presence of the *fdhF* mRNA hairpin, promoting selenocysteine incorporation into formate dehydrogenase H in *E.coli*, results in a strongly reduced binding of *fdhF* mRNA to the ribosome when the hairpin is placed within the ribosomal mRNA binding track. By toeprint or footprint analysis the 'entry site' of the ribosomal mRNA track has been shown to be located between +16 and +19 bases away from the first base of the P-site codon (5,26). An up to 25-fold reduction in binding is observed of those mRNA constructs which place the mRNA hairpin closer than ~16 bases to the ribosomal P-site (Fig. 6). Thereby, the AH84 $[UUC]_3$ and AH81 $[UUC]_2$ mRNA constructs resulted in the strongest decrease in binding to 30S subunits, while, in comparison, binding of AH75 $[UUC]_0$ and AH78 $[UUC]_1$ mRNAs was slightly higher. A possible explanation would be that, assuming an A-helical conformation of the *fdhF* mRNA within the ribosomal mRNA track, the *fdhF* hairpin might inhibit binding to the ribosome differently dependent on which side of the mRNA helix the hairpin is located.

Within the AH75 $[UUC]_0$ mRNA–ribosome complex a second toeprint signal at position +18 in addition to the one at position +16 is observed. The two toeprint signals can be rationalised by postulating that $tRNA^{fMet}$ binds to the AH75 $[UUC]_0$ mRNA within two different reading frames; the +16 toeprint being due to decoding of $tRNA^{fMet}$ at AUG-UGA, the +18 toeprint due to decoding of $tRNA^{fMet}$ at AUG-UGA. Since the optimal spacing between the Shine–Dalgarno sequence and the start codon was shown to be seven rather than five bases, as used in our mRNA constructs (27), binding of $tRNA^{fMet}$ preferentially to the GUG

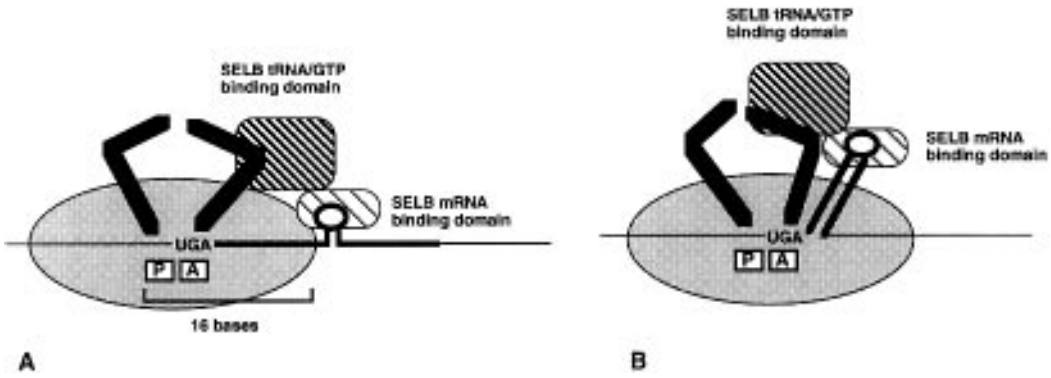

Figure 7. Two models of possible modes of interaction of the *fdhF* mRNA hairpin with the ribosome. (**A**) The *fdhF* mRNA hairpin becomes partly unfolded prior to or while entering the ribosomal mRNA track. (**B**) The *fdhF* mRNA hairpin is able to be accomodated by the ribosomal mRNA track and is therefore present completely folded during decoding of the UGA selenocysteine codon by $tRNA^{Sec}$ (23). mRNA and tRNA/GTP binding domains of SELB are indicated, the *fdhF* hairpin structure is shown in bold.

rather than to the AUG codon would result in a seven base spacing. As all other mRNA constructs used in our study do not contain the UGA immediately adjacent to the AUG codon, only one toeprint signal is observed at position +16. Accordingly, the +18 toeprint cannot be used as a means to compare binding of mRNA constructs to 30S ribosomal subunits.

To test for translation *in vivo*, a DNA fragment resembling AH75 $[UUC]_0$ mRNA was fused in-frame 5′ of the *lacZ* gene lacking its translation initiation region. *In vivo* expression of the resulting plasmid, pATG-TGA, showed a dramatic decrease in β-galactosidase synthesis. However, introducing a spacer region of seven codons between the AUG and UGA codon restored read-through of the UGA codon to wild-type levels. This is consistent with the *fdhF* hairpin preventing translation of the mRNA when placed adjacent to the AUG initiation codon.

In the presence of $tRNA^{Sec}$ we could not observe any ribosome-dependent toeprint signals in our mRNA constructs, indicative of the mRNA hairpin interfering with decoding of the UGA codon by $tRNA^{Sec}$. This could be due to either steric hindrance by the *fdhF* hairpin structure or the fact that the UGA codon might be embedded within a secondary structure and thereby unable to base pair with the anticodon of $tRNA^{Sec}$. Chemical probing data demonstrate (Fig. 5 and data not shown) that the selenocysteine codon is accessible to base-specific probes. Therefore, steric hindrance is the most likely explanation for the absence of any toeprint signals in the presence of $tRNA^{Sec}$. In fact, deletion of the hairpin structure 3′ of the UGA codon within AH75 $[UUC]_0$ mRNA results in a +16 toeprint signal in the presence of $tRNA^{Sec}$ (23).

Since toeprinting studies only show a low resolution picture of how mRNAs interact with the ribosome, we tried to elucidate the mechanism of interaction of the AH75 $[UUC]_0$ mRNA with the ribosome by footprint analysis with chemical probes. By this approach we show that the AH75 $[UUC]_0$ mRNA is not unfolded by 30S ribosomal subunits (Fig. 5). Despite the presence of $tRNA^{fMet}$, we did not observe protection of the G base of the AUG initiation codon, as was shown previously for gene 32 mRNA (5). Although we have no direct evidence for binding of $tRNA^{fMet}$ to 30S subunits, lack of interaction with the AUG codon might be indicative of the presence of a 'pre-ternary complex', where the tRNA is bound to the ribosome but not engaged in a codon–anticodon interaction (28–30). Lack of codon–anticodon interaction at the P-site codon preceeding the UGA selenocysteine codon might be indicative of the *fdhF* mRNA hairpin interfering with decoding. Therefore, this might be consistent with a model where partial unfolding of the hairpin structure has to occur prior to the UGA codon entering the ribosomal A-site.

Our results are at variance with a model suggested by Ringquist *et al.* (23), which implies that the ribosomal mRNA binding track is able to accomodate even large mRNA structures. This model is based on the presence of an 'extended' toeprint signal 36 bases downstream of the +16 signal within an AUG-UGA-fdhF hairpin mRNA. We also observed this extended toeprint, but only when we used the shortest construct (AH75 $[UUC]_0$ mRNA), containing the stem–loop structure immediately next to the AUG initiation codon. However, within all other mRNA constructs (e.g. AH78 $[UUC]_1$–AH90 $[UUC]_5$ mRNAs), which extended the spacing between the initiation codon and the stem–loop structure by one codon each, did not result in an extended toeprint (Figs 2 and 6). One possible explanation for the presence of the extended toeprint within AH75 $[UUC]_0$ mRNA only is a conformational change within the mRNA upon binding to the ribosome. This conformational change could stall the reverse transcriptase at the extended toeprint position. In fact, an increase in the reactivity of base G+52, located adjacent to the extended toeprint signal, towards KE was observed upon binding of the mRNA to 30S ribosomal subunits, indicative of some structural rearangement occuring within AH75 $[UUC]_0$ mRNA (Fig. 5).

In conclusion, our study shows that: (i) the *fdhF* mRNA secondary structure interferes with binding of the mRNA to the ribosome *in vitro* and prevents translation *in vivo* when placed within the ribosomal mRNA binding track; (ii) an extended toeprint signal, indicative of large structured mRNAs being accomodated by the ribosomal mRNA track, is missing in all but one mRNA construct with $tRNA^{fMet}$ as the initiator tRNA; (iii) although bases of the UGA codon are accessible to chemical probes, decoding by $tRNA^{Sec}$ is sterically hindered due to the presence of the mRNA stem–loop structure.

Taken together these data indicate that the *fdhF* hairpin has to be unfolded during elongation prior to entering the ribosomal mRNA track *in vivo*, as the mRNA hairpin interferes with binding of the mRNA to the ribosome, as well as with decoding of selenocysteinyl-$tRNA^{Sec}$ at the UGA codon. However, since defined initiation complexes were used to mimic distinct steps of

elongation, it still has to be demonstrated that the mode of interaction of an mRNA with the ribosome is similar during the initiation and elongation phases. In addition, we omitted any initiation or elongation factors in our toeprinting assays, which might affect binding of mRNAs to the ribosome (30). In that respect, our study can only be a first approach in trying to elucidate the interaction of the structured *fdhF* mRNA with the ribosome.

The consequences of our model would imply that the *fdhF* hairpin is partially unfolded during decoding of the UGA selenocysteine codon by selenocysteinyl-tRNA^{Sec}, as the UGA codon is adjacent to the stem of the *fdhF* hairpin (Fig. 1). Would this interfere with binding of special elongation factor SELB to the *fdhF* hairpin during decoding? Special elongation factor SELB was shown to be structurally divided into an N-terminal domain, which shares extensive sequence homology with EF-Tu and binds to selenocysteinyl-tRNA^{Sec} and GTP, and a separate C-terminal domain, required for binding exclusively to only the upper half of the mRNA hairpin, including the loop region (23,31). Therefore, we propose a model where the upper part of the *fdhF* mRNA hairpin which binds to the C-terminus of SELB can be placed 'outside' the ribosomal mRNA track during decoding of the UGA codon (Fig. 7A). Upon decoding of the UGA codon by selenocysteinyl-tRNA^{Sec}, SELB might dissociate from the mRNA hairpin, whereupon the mRNA secondary structure could be unfolded by the elongating ribosome. It will be interesting to investigate whether other mRNA secondary structures, like for example pseudoknots, are also unfolded by the ribosome prior to entering the ribosomal mRNA track.

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