

# EF-G-catalyzed translocation of anticodon stem-loop analogs of transfer RNA in the ribosome

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**Translocation, catalyzed by elongation factor EF-G, is the precise movement of the tRNA–mRNA complex within the ribosome following peptide bond formation. Here we examine the structural requirement for A- and P-site tRNAs in EF-G-catalyzed translocation by substituting anticodon stem-loop (ASL) analogs for the respective tRNAs. Translocation of mRNA and tRNA was monitored independently; mRNA movement was assayed by toeprinting, while tRNA and ASL movement was monitored by hydroxyl radical probing by Fe(II) tethered to the ASLs and by chemical footprinting. Translocation depends on occupancy of both A and P sites by tRNA bound in a mRNA-dependent fashion. The requirement for an A-site tRNA can be satisfied by a 15 nucleotide ASL analog comprising only a 4 base pair (bp) stem and a 7 nucleotide anticodon loop. Translocation of the ASL is both EF-G- and GTP-dependent, and is inhibited by the translocational inhibitor thiostrepton. These findings show that the D, T and acceptor stem regions of A-site tRNA are not essential for EF-G-dependent translocation. In contrast, no translocation occurs if the P-site tRNA is substituted with an ASL, indicating that other elements of P-site tRNA structure are required for translocation. We also tested the effect of increasing the A-site ASL stem length from 4 to 33 bp on translocation from A to P site. Translocation efficiency decreases as the ASL stem extends beyond 22 bp, corresponding approximately to the maximum dimension of tRNA along the anticodon-D arm axis. This result suggests that a structural feature of the ribosome between the A and P sites, interferes with movement of tRNA analogs that exceed the normal dimensions of the coaxial tRNA anticodon-D arm.**

**Keywords:** EF-G/mRNA/ribosome/translocation/tRNA

## Introduction

Translocation is the precise, coordinated movement of the two tRNAs and their associated mRNA by one codon following peptide bond formation (Spirin, 1985; Liljas *et al.*, 1995; Czworkowski and Moore, 1996). In bacteria, this reaction is catalyzed by elongation factor EF-G in a GTP-dependent fashion (Kaziro, 1978) and requires occupancy of both the ribosomal A and P sites by tRNA (Lucas-Lenard and Haenni, 1969; Ishitsuka *et al.*, 1970; Roufa *et al.*, 1970; Modolell *et al.*, 1973; Holschuh *et al.*,

1980). According to the hybrid states model, translocation of tRNAs on the ribosome occurs in two separable steps (Moazed and Noller, 1989). In the first step, movement of the acceptor ends of tRNAs relative to the 50S ribosomal subunit occurs spontaneously following peptide bond formation, independent of EF-G and GTP. The second step, which is EF-G- and GTP-dependent, results in translocation of the anticodon arms of the two tRNAs, together with their associated mRNA, relative to the 30S subunit. Therefore, the main thermodynamic barrier to the step of translocation that is catalyzed by EF-G may be to overcome interactions between the 30S subunit and the anticodon arm of tRNA.

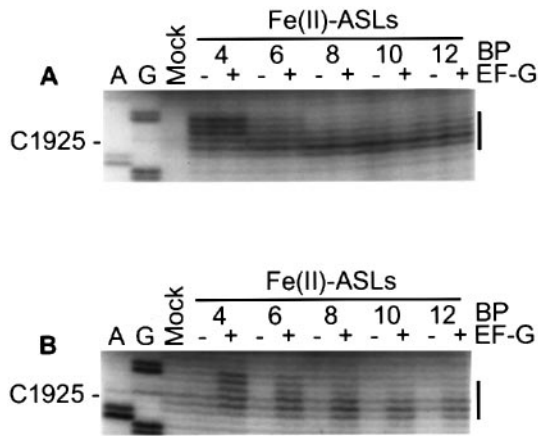
We therefore asked whether EF-G can catalyze translocation of the anticodon stem-loop of tRNA, independent of the rest of the tRNA structure. Pre-translocation complexes were constructed by substituting one of the two tRNAs with an anticodon stem-loop (ASL) tRNA analog bound in a mRNA-specific fashion to the ribosomal A or P site, and assaying translocation using two independent methods. The movement of mRNA was monitored by toeprinting (Hartz *et al.*, 1988, 1989), while the coupled movement of tRNA or ASL was detected by directed hydroxyl radical probing using Fe(II)-ASL (Joseph *et al.*, 1997) and by chemical footprinting (Moazed and Noller, 1986). We find that translocation depends on occupancy of both A and P sites. The requirement for an A-site tRNA can be satisfied by an ASL, while no translocation occurs if the P-site tRNA is substituted with an ASL. In addition, we tested translocation efficiency as a function of increasing A-site ASL stem length. Our results indicate that translocation efficiencies of ASLs with stems >22 bp are increasingly inhibited, suggesting that some feature of the ribosome blocks the movement of ASLs with stems that extend significantly beyond the elbow region of tRNA.

## Results

### **Construction of defined pre-translocation complexes**

We used a series of ASL analogs of tRNA, of increasing stem lengths, starting with a 4 bp stem and extending to 33 bp by increments of 2–3 bp (Joseph *et al.*, 1997). The ASLs are based on the yeast tRNA<sup>Phe</sup> sequence, and so bind specifically to ribosomes programmed with the codon UUU. Pre-translocation complexes were constructed using ribosomes programmed with a 142 nucleotide fragment of phage T4 gene 32 mRNA carrying a deacylated tRNA<sub>f</sub><sup>Met</sup> in the P site and either a deacylated tRNA<sup>Phe</sup> or an ASL in the A site. Movement of mRNA was assayed by toeprinting (Hartz *et al.*, 1988, 1989) and coupled movement of tRNA or ASL was monitored by directed hydroxyl radical probing from Fe(II) tethered to its 5' end



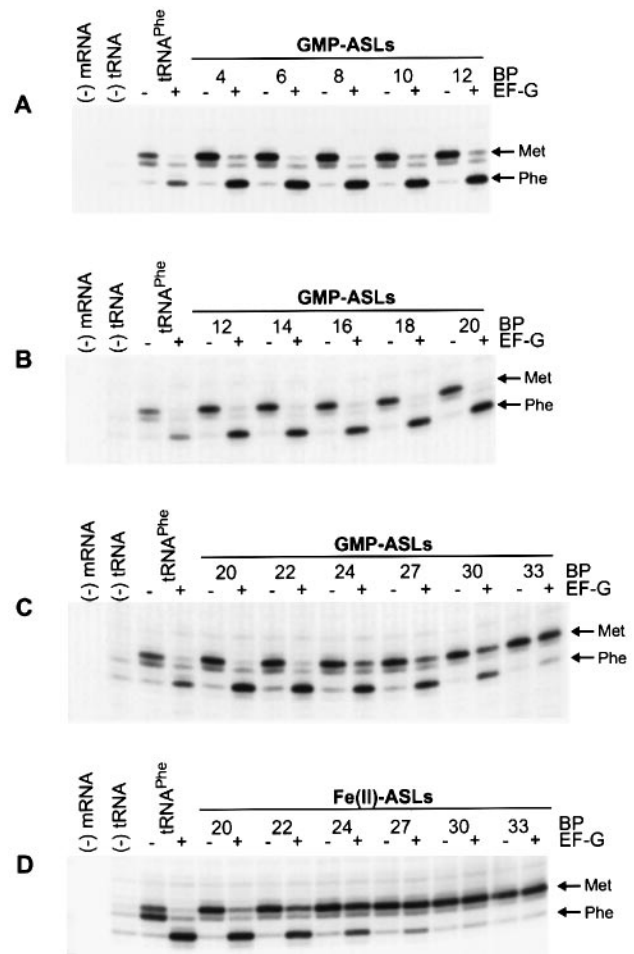


**Fig. 3.** Translocation of ASLs assayed by directed hydroxyl radical probing. (A) Cleavage pattern obtained around position 1925 in 23S rRNA when Fe(II)-ASL4–12 are bound directly to the 30S P site of 70S ribosomes. (B) Translocation of Fe(II)-ASL4–12 from the A site to P site by EF-G results in the appearance of the P-site specific cleavage pattern. A and G, dideoxy sequencing lanes; Mock, probing in the absence of ASL.

In some cases, we observe a doublet corresponding to the toeprint of the pre-translocation complex. This is seen for the complex containing  $tRNA_f^{Met}$  or  $ASL6^{Met}$  in the P site and  $tRNA^{Phe}$  in the A site (Figure 2A and C), but not for the other complexes, and is more pronounced in the case of  $tRNA^{Phe}$  yeast than for  $tRNA^{Phe}$  *Escherichia coli* (data not shown). The reason for the appearance of this doublet is not known, but may be to do with a slightly different orientation of these tRNA–mRNA complexes with respect to the ribosome.

#### Translocation monitored by tRNA or ASL movement

We also assayed translocation by monitoring the repositioning of tRNA or ASLs in their ribosomal binding sites. One way in which we monitored the position of a tRNA or ASL was to monitor changes in its surrounding rRNA environment, using directed hydroxyl radical probing from Fe(II) tethered to its 5' end. Characteristic sets of nucleotides are cleaved by hydroxyl radicals when Fe(II)-ASLs are bound to the ribosomal A or P sites (Joseph *et al.*, 1997). These patterns can be used to monitor the locations of ASLs in the ribosome. Fe(II)-ASLs cause cleavage of nucleotides in the 1925 region of domain IV of 23S rRNA when bound to the P site (Figure 3A; Joseph *et al.*, 1997). The different cleavage patterns obtained with the different ASLs reflect changes in the rRNA environment surrounding the tethered Fe(II) as a function of increasing stem length (Joseph *et al.*, 1997). This cleavage pattern is unaffected by addition of EF-G and GTP, indicating that P site-bound Fe(II)-ASL4–12 does not translocate (Figure 3A). Pre-translocation complexes containing Fe(II)-ASLs bound to the A site do not cleave the 1925 region of 23S rRNA (Joseph *et al.*, 1997; Figure 3B, –EF-G lanes). However, addition of EF-G–GTP to these pre-translocation complexes results in appearance of the characteristic P-site cleavage pattern, indicating that EF-G catalyzed translocation of ASLs from A to P site (Figure 3B, +EF-G lanes). Appearance of characteristic P-site hydroxyl radical cleavage patterns at



**Fig. 4.** Effect of A-site ASL stem length on translocation. (A) GMP-ASL4–12, (B) GMP-ASL12–20, (C) GMP-ASL20–33 and (D) Fe(II)-ASL20–33. Labels are as described in Figure 2.  $tRNA^{Phe}$ , reaction in the presence of  $tRNA^{Phe}$  as A-site substrate.

other positions in 23S, 16S and 5S rRNAs confirmed EF-G-catalyzed movement of these Fe(II)-ASLs from A to P site (data not shown).

ASL translocation was also tested by chemical footprinting. Binding of tRNA or ASLs to the A site protects characteristic bases, including nucleotide residues A1492 and A1493, in 16S rRNA from chemical probes (Moazed and Noller, 1986). Translocation results in loss of protection of A1492 and A1493 due to the movement of tRNA from A to P site (Moazed and Noller, 1989). Using this assay, translocation was observed for ASL4, but not for ASL30 or 33 (data not shown; see following section). Movement of the ASL into the P site could not be monitored directly by footprinting, because the 30S P site is occupied both before and after translocation.

#### Dependence of translocation on ASL stem length

We monitored the effect of further increasing the length of the ASL stem on translocation using the toeprint assay. Efficient EF-G-dependent translocation is supported by ASL stem lengths ranging from 4 to >20 bp (Figure 4). However, there is increasing inhibition of translocation for ASLs longer than 22 bp (Figure 4C). In addition, we monitored the ability of 5'-Fe(II)-BABE-derivatized ASLs to support translocation using the toeprint assay. These

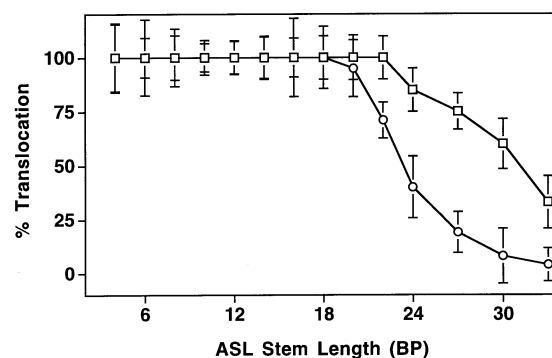
derivatized ASLs also supported translocation, but inhibition was observed at slightly shorter ASL stem lengths than for non-derivatized ASLs (Figure 4D), probably due to the added bulk and/or charge of the Fe(II)-BABE moiety. Although Fe(II)-ASL4–20 translocates efficiently, translocation of Fe(II)-ASL24–33 is strongly reduced compared with that of unmodified ASL24–33 (Figures 4D and 5). Thus, while ASLs with stems up to 20 bp in length are translocated efficiently, ASLs with longer stems are increasingly impaired in their ability to translocate from A to P site.

## Discussion

According to the hybrid states model for translocation of tRNAs on the ribosome, movement of tRNA and mRNA in the 30S subunit can be uncoupled from movement of tRNA in the 50S subunit (Moazed and Noller, 1989). Furthermore, interactions between tRNA and the 30S subunit are believed to involve only the ASL (Rose *et al.*, 1983; Moazed and Noller, 1986; Hüttenhofer and Noller, 1992). Therefore, EF-G-dependent translocation is predicted to involve primarily rearrangements of the interactions between the anticodon ends of the tRNAs and their respective binding sites on the 30S subunit. Our finding, that even a 4 bp ASL can be translocated efficiently from A to P site, supports this prediction. The lack of a requirement for the rest of the A-site tRNA structure, including the elbow region and the D, T and acceptor stems, is remarkable. This is especially so when one considers that these elements of A-site tRNA are believed to be nearest to those features of the 50S subunit that have traditionally been identified with the mechanism of translocation, including the L7/L12 stalk, the sarcin/ricin and thiostrepton loops of 23S rRNA, and the binding site for EF-G (Möller, 1990). These considerations call our attention to the potential role of the 30S subunit in translocation, particularly for A-site tRNA. Translocation of tRNAs can also occur in the absence of mRNA (Belitsina *et al.*, 1981, 1982) which implies that movement of tRNA is the primary event in translocation, while mRNA may be translocated passively via codon–anticodon interactions.

In contrast, ASLs do not support translocation when substituted for P-site tRNA, possibly reflecting a requirement for interactions involving the acceptor end of deacylated tRNA. Previous studies have shown that the rate of translocation is inhibited by up to 40-fold if A76 of P-site tRNA is deleted or chemically modified, reducing E-site binding (Lill *et al.*, 1989). This could mean that interactions between the acceptor stem of P-site tRNA and the 50S subunit E site (in the P/E state) are required before translocation of the anticodon ends of the tRNAs with respect to the 30S subunit can take place. Preliminary experiments indicate that supplying the acceptor stem of tRNA *in trans* is insufficient to promote translocation in ribosomes containing a P-site ASL, and either an ASL or full-length tRNA in the A site (S. Joseph and H.F. Noller, unpublished).

Previously we have shown that all of these ASL analogs bind to intact 70S ribosomes in a mRNA-dependent manner to both A and P sites (Joseph *et al.*, 1997). In spite of extended stem lengths that exceed the maximum



**Fig. 5.** Translocation efficiency as a function of increasing A-site ASL stem length (BP). Squares, unmodified GMP-ASLs; circles, Fe(II)-ASLs. Values are normalized with respect to translocation of control tRNA<sup>Phe</sup>, set to 100%; the actual extent of translocation of control tRNA<sup>Phe</sup> varied from 70 to 80%.

dimension of tRNA by  $>20 \text{ \AA}$ , ASLs ranging in stem length from 4 to 33 bp all showed normal A- and P-site footprints on 16S rRNA that were indistinguishable from those of intact tRNA. ASLs with stems  $>20$  bp extend beyond the envelope of the tRNA structure, near the elbow region. This indicates that either the ribosome is unobstructed in the space projecting directly above the elbow of both A- and P-site tRNAs, or that the tRNA–ribosome complex is flexible enough to accommodate the extended ASL structures. Even though ASL22–33 appear to bind normally to the 30S ribosomal A site, their translocation efficiency is increasingly impaired as a function of stem length (Figure 5). A simple interpretation of this result is that some feature of ribosomal structure blocks their movement. According to this view, the ASLs reflect the minimum depth of the channel traversed by tRNA as it moves from A to P site of the ribosome. This would imply that there is a close fit between some feature of the ribosome and the elbow of tRNA at some intermediate point during translocation. Recent electron microscopy reconstruction experiments have localized the positions of tRNA in the ribosome at low resolution (Agrawal *et al.*, 1996; Stark *et al.*, 1997). The results of these studies suggest that the barrier suggested by our experiments is likely to be the central protuberance of the 50S subunit. Alternatively, binding interactions between the ends of the longer ASLs and some feature of the large subunit could interfere with translocation. Another interpretation is that binding of the longer ASLs may distort the ribosome structure, which in turn could inhibit translocation.

In summary, the minimal molecular components required for EF-G-dependent translocation, under *in vitro* conditions, can be reduced to a 4 bp ASL in the A site, and a full-length deacylated tRNA in the P site, in addition to the 70S ribosome, mRNA, EF-G and GTP. Although mRNA was previously shown not to be essential (Belitsina *et al.*, 1981, 1982), it is required in our system in order to bind the A-site ASL. The structural basis for the different specific requirements for the A- and P-site tRNA substrates is unknown, but places significant constraints on possible molecular mechanisms for translocation.

## Materials and methods

### *In vitro* transcription of gene 32 mRNA and ASLs

A gene 32 mRNA fragment corresponding to positions -57 to +85 was transcribed from a DNA template generated by PCR amplification of plasmid pRS170 (Hartz *et al.*, 1989) using T7/-40 and AL2 primers essentially as previously described (Hüttenhofer and Noller, 1994). The mRNA was purified on a 10% denaturing polyacrylamide gel and recovered as previously described (Hüttenhofer and Noller, 1994).

ASL analogs of tRNA were transcribed *in vitro* using T7 RNA polymerase and the corresponding oligodeoxynucleotide templates as previously described (Joseph *et al.*, 1997). A new transcription template was used for the synthesis of ASL6<sup>Met</sup> (RNA: 5'-GGCAGGCU-CAUAACCGCC-3'), the 6 bp ASL analog of tRNA<sup>Met</sup>. GMP (or 5'-guanosine- $\alpha$ -phosphorothioate, GMPS) was introduced at the 5' end of the ASLs by transcribing in the presence of a 5-fold molar excess of GMP (or GMPS) over each NTP essentially as previously described (Joseph and Noller, 1996; Joseph *et al.*, 1997). The 5' GMPS-ASLs were derivatized with Fe(II)-BABE (DeRiemer *et al.*, 1981; Rana and Meares, 1990) and purified as previously described (Joseph *et al.*, 1997).

### Binding of ASLs to 70S ribosomes and the translocation reaction

ASLs were specifically bound to the ribosomal A site by using ribosomes programmed with gene 32 mRNA containing tRNA<sup>Met</sup> as previously described (Joseph *et al.*, 1997). Briefly, 10 pmol (0.4  $\mu$ M) tight couple 70S ribosomes were activated by incubating for 10 min at 42°C and 10 min at 37°C in binding buffer (80 mM K-cacodylate pH 7.2, 20 mM Mg(OAc)<sub>2</sub>, 150 mM NH<sub>4</sub>Cl) followed by addition of 20 pmol (0.8  $\mu$ M) gene 32 mRNA and incubation for 6 min at 37°C. Next, 12 pmol (0.48  $\mu$ M) *E. coli* tRNA<sup>Met</sup> (Subriden) was added and the complexes were incubated for 10 min at 37°C followed by addition of either 12 pmol (0.48  $\mu$ M) *E. coli* tRNA<sup>Phe</sup> (Subriden) or 150 pmol (6.0  $\mu$ M) ASL and incubation for 30 min at 37°C and for 30 min on ice. The translocation reaction was performed by addition of 50 pmol (2  $\mu$ M) EF-G and 300  $\mu$ M GTP (final concentration) to the complexes (25  $\mu$ l final volume) and incubating at 37°C for 20 min. All reagents were made up in binding buffer before addition. For binding Fe(II)-BABE-ASLs directly to the ribosomal P site, *E. coli* tRNA<sup>Met</sup> was omitted from the translocation reaction mixture. In some experiments, minor variations from the above standard procedure were followed as follows. (i) GTP-dependence and thiostrepton inhibition of translocation: to remove trace amounts of GTP present in the EF-G preparation, EF-G (30  $\mu$ M) was preincubated with 70S ribosomes (6  $\mu$ M) programmed with poly(U) mRNA (1  $\mu$ g/ $\mu$ l) for 10 min at 37°C in binding buffer to hydrolyze the contaminating GTP before adding to the pre-translocation complexes as described above. For thiostrepton inhibition, pre-translocation complexes were incubated with thiostrepton (0.1 mM) for 10 min at 37°C prior to addition of EF-G and GTP to the reaction. Thiostrepton (0.1 mM) was also present in the subsequent toeprinting reaction (see below). (ii) Binding of ASLs to P site and translocation: in experiments where ASLs were bound directly to the ribosomal P site, the concentrations of all the components were identical to the standard condition except 50 pmol (2  $\mu$ M) ASLs were used for formation of the pre-translocation complex.

### Toeprinting assays

For toeprinting, gene 32 mRNA annealed to 5' <sup>32</sup>P-end-labeled AL2 primer as previously described (Hartz *et al.*, 1988) was used to program ribosomes in the translocation reaction described above. After translocation, 1  $\mu$ l aliquots of the reaction mixture were used for toeprinting assays. End-labeled AL2 primer, which is complementary to positions +64 to +85 of gene 32 mRNA, was extended using 1 unit of AMV reverse transcriptase in the presence of 375  $\mu$ M each of the four dNTPs, 10 mM Tris pH 7.5, 10 mM Mg acetate, 60 mM ammonium chloride, 6 mM  $\beta$ -mercaptoethanol (10  $\mu$ l final volume), at 37°C for 15 min. A and G dideoxy sequencing reactions were carried out in parallel, as described above. The reactions were stopped by addition of 10  $\mu$ l of loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and heating to 90°C for 2 min. Toeprint reactions were analyzed on 10% denaturing polyacrylamide gels. The gels were quantified using a Molecular Dynamics PhosphorImager.

### Fe(II)-BABE-ASL probing

Directed hydroxyl radical probing using Fe(II)-BABE-derivatized ASLs were performed after translocation essentially as described (Joseph and Noller, 1996; Joseph *et al.*, 1997). Briefly, hydroxyl radical strand scission

was initiated by the addition of ascorbate (5 mM final concentration) and hydrogen peroxide (0.05% final concentration) to 25  $\mu$ l of the translocation reaction mixture (see above) followed by incubation for 10 min at room temperature. Reactions were stopped by addition of 300  $\mu$ l of cold ethanol and 3M Na acetate (0.3 M final concentration) and quick-freezing in a dry-ice-ethanol bath. Extraction of rRNAs from all samples, primer extension reactions and gel electrophoresis were carried out as previously described (Stern *et al.*, 1988).

### Chemical probing

Chemical probing (Stern *et al.*, 1988) of translocation reactions was performed by the addition of 2  $\mu$ l DMS (1:10 dilution in 95% ethanol) to 25  $\mu$ l of translocation reaction mixture (see above) followed by incubation at 37°C for 10 min. Reactions were stopped by the addition of 25  $\mu$ l DMS stop solution (1 M Tris-HCl pH 7.5, 1 M  $\beta$ -mercaptoethanol, 0.1 M EDTA), 300  $\mu$ l cold ethanol and 3M Na acetate (0.3 M final concentration) and quick-freezing in a dry-ice-ethanol bath. The samples were extracted and primer extension reactions carried out as described (see above).

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