Ribose 2'-hydroxyl groups in the 5' strand of the acceptor arm of P-site tRNA are not essential for EF-G catalyzed translocation

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ABSTRACT

The coupled movement of tRNA–mRNA complex through the ribosome is a fundamental step during the protein elongation process. We demonstrate that the ribosome will translocate a P-site-bound tRNA^{Met} with a break in the phosphodiester backbone between positions 17 and 18 in the D-loop. Crystallographic data showed that the acceptor arms of P- and E-site tRNA interact extensively with the ribosomal large subunit. Therefore, we used this fragmented P-site-bound tRNA^{Met} to investigate the contributions of single 2'-hydroxyl groups in the 5' strand of the acceptor arm for translocation into the ribosomal E-site. EF-G-dependent translocation of the tRNAs was monitored using a toeprinting assay and a fluorescence-based rapid kinetic method. Surprisingly, our results show that none of the 2'-hydroxyl groups in the 5' strand of the acceptor arm of P-site-bound tRNA^{Met} between positions 1–17 play a critical role during translocation. This suggests that either these 2'-hydroxyl groups are not important for translocation or they are redundant and the three-dimensional shape of the P-site tRNA is more important for translocation.

Keywords: ribosome; translocation; tRNA; mRNA; EF-G; mechanism

INTRODUCTION

The ribosome is a macromolecular machine composed of a large and a small subunit that has binding sites for three transfer ribonucleic acids (tRNAs), the aminoacyl site (A-site), the peptidyl site (P-site), and the exit site (E-site). The acceptor arm of the tRNAs interacts with the large ribosomal subunit, while the anticodon arm of the tRNAs interacts with the small ribosomal subunit and with the messenger RNA (mRNA). During the elongation cycle of protein synthesis, tRNAs iteratively move from the A-site to the P-site and then to the E-site, making specific contacts within each ribosomal site. Movement of the tRNAs and the associated mRNA is catalyzed by an elongation factor (EF-G in *Escherichia coli*) and requires GTP hydrolysis (Rodnina et al. 1997). Recent X-ray crystal structures of the ribosome showed that the mRNA–tRNA complex makes extensive interactions with the large and small ribosomal subunits

(Carter et al. 2000; Ogle et al. 2001; Yusupov et al. 2001). Translocation, therefore, must involve disruption of specific contacts in one site while establishing new contacts in the next site as the tRNAs move from one site to another within the ribosome. However, the interactions between the tRNAs and the ribosome that are critical for translocation, as well as how the ribosome orchestrates this complex process, are not yet clear. Previous studies have demonstrated that the ribosome is capable of translocating an anticodon stem-loop analog (ASL) of tRNA containing a 7-nt anticodon loop and a 4-bp stem from the ribosomal A-site to the ribosomal P-site (Joseph and Noller 1998). More recent studies have identified 2'-hydroxyl groups and non-bridging phosphate oxygen atoms within the A-site ASL that are important for translocation into the P-site (Phelps et al. 2002; Phelps and Joseph 2005). This shows that specific contacts between the backbone of the ASL and the 30S subunit are essential for translocation.

Interestingly, the ribosome is unable to translocate an ASL from the ribosomal P-site to the E-site (Joseph and Noller 1998). This illustrates that although the tRNA D-loop, TΨC-loop, and acceptor arm are not essential for EF-G-dependent translocation of tRNA from the ribosomal A-site to the P-site, they are required for its movement from

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the P-site to the E-site (Joseph and Noller 1998). Previously, we demonstrated that the ribosome will bind and translocate a tRNA having a nick in its TΨC-loop from the P-site (Feinberg and Joseph 2001). Using this nicked tRNA, we identified two 2'-hydroxyl groups at positions 71 and 76 in the 3' strand of the acceptor arm of the P-site tRNA that are essential for translocation (Feinberg and Joseph 2001). These 2'-hydroxyl groups interact with the 23S rRNA in the ribosomal E-site, establishing their role in translocation. Using a similar approach, we now investigated the contribution of the ribose 2'-hydroxyl groups in the 5' strand of the acceptor arm of P-site tRNA in translocation. We demonstrate that the ribosome is capable of translocating a tRNA with a break in the D-loop from the P-site to the E-site. We synthesized a library of tRNAs having modifications at their ribose 2'-hydroxyl groups between positions 1–17 and assayed their ability to translocate from the P-site to the E-site. Using both toeprinting assays (Hartz et al. 1988; Joseph and Noller 1998) and our recently described fluorescence-based kinetic assay (Studer et al. 2003), we systematically determined the contribution of individual 2'-hydroxyl groups to translocation of the tRNA from the ribosomal P-site. Interestingly, we find that none of these positions, when modified with a single 2'-deoxy nucleotide, have an adverse affect on translocation. These results suggest that the three-dimensional shape of the P-site tRNA is more important for translocation than specific contacts between the ribose 2'-hydroxyl groups at positions 1–17 and the ribosome.

RESULTS

Folding of fragmented tRNA^{Met}

In order to study the role of the 5' strand of the acceptor arm of P-site tRNA^{Met} in translocation, we used an *E. coli* tRNA^{Met} with a break in the D-loop between positions 17 and 18. This fragmented tRNA was assembled from two separate fragments (Fig. 1A), a large 3'-fragment containing bases 18–76 of tRNA^{Met} and a smaller 5'-fragment containing bases 1–17. Analysis by nondenaturing polyacrylamide gel electrophoresis showed that the large 3'-fragment by itself migrates as two distinct conformers (Fig. 1B). One of the conformers migrates more rapidly and the other conformer migrates slower than the folded full-length tRNA^{Met}. However, in the presence of the smaller 5'-fragment, the large 3'-fragment folds into a conformation that comigrates with the full-length tRNA^{Met} (Fig. 1B). In addition, 5' fragments incorporating single 2'-deoxy group substitutions at positions 1–17 also anneal to the 3' fragment and fold into the tRNA tertiary structure (Fig. 1B; data not shown). Thus, a nick in the backbone between positions 17 and 18 and single 2'-deoxy substitutions at positions 1–17 does not prevent the proper folding of the tRNA.

FIGURE 1. Folding of fragmented *E. coli* tRNA^{Met}. (A) Secondary structure of tRNA^{Met} showing the nick in the phosphodiester backbone between positions 17 and 18. (B) Folding of fragmented tRNA^{Met} analyzed by nondenaturing PAGE. (Lane tRNA^{Met}) *E. coli* tRNA^{Met} transcript, (lane RNA 1–17) unsubstituted smaller RNA fragment, (lane RNA 1–17 (2'-d1)) small RNA fragment with a single 2'-deoxy substitution at position 1. Plus (+) and minus (–) signs indicate presence and absence, respectively, of the 3' large fragment (positions 18–76). Mobility of folded tRNA^{Met}, 5' small fragment, and 3' large fragment is indicated on the right.

Fragmented tRNA^{Met} is translocated from the ribosomal P-site

Pretranslocation complexes were formed by specifically binding either a deacylated tRNA^{Met} or fragmented tRNA^{Met} in the ribosomal P-site and an ASL4^{Phe} in the ribosomal A-site. ASL4^{Phe} was used because translocation with a full-length tRNA^{Phe} is very rapid and the linear range of translocation cannot be easily resolved using the toeprinting assay (Studer et al. 2003). In addition, we used a conventional buffer system that further reduces the rate of translocation. Toeprinting assays demonstrated that tRNA^{Met} assembled from an in vitro transcribed 3'-large fragment and a synthetic 5'-small fragment was translocated from the ribosomal P-site (Fig. 2A). However, consistent with previous studies (Rose et al. 1983; Moazed and Noller 1986; Feinberg and Joseph 2001), the large 3'-fragment 18–76 containing the sequence corresponding to the anticodon loop of tRNA^{Met} was capable of binding to the 30S subunit P-site even in the absence of the 5'-fragment, but was not translocated into the E-site (Fig. 2A).

Replacement of the 5'-small fragment with a fully substituted 2'-deoxyribose analog or 2'-O-methyl analog inhibits translocation (Fig. 2A; data not shown). Fragmented tRNA with the fully 2'-deoxy-substituted 5'-small fragment is more severely inhibited than is the 2'-O-methyl-substituted tRNA. Native gel analysis showed that the fully 2'-deoxyribose-substituted 5'-fragment, when annealed to the 3'-large fragment, does not fold into a proper tRNA conformation (data not shown). It is known that a 2'-deoxy substitution favors the C_{2'}'-endo sugar conformation while the 2'-O-methyl substitution retains the C_{3'}'-endo

FIGURE 2. Translocation of fragmented tRNA^{Met} from the ribosomal P-site. (A) Translocation of fragmented tRNA^{Met} having either unsubstituted or fully 2'-O-methyl-substituted small fragment. (Lane (-) RNA 1-17) Absence of the 5' small fragment, (lane RNA 1-17) presence of unsubstituted 5' small fragment, (lane 2'-O-me 1-17) presence of fully 2'-O-methyl-substituted small fragment. Minus (-) and plus (+) signs indicate absence or presence of EF-G. Arrows indicate toeprints before (Pre) and after (Post) translocation. (B) Translocation of fragmented tRNA^{Met} having single 2'-deoxy substitutions at positions 1-17 in the small fragment. Lanes as indicated above. (Lane tRNA^{Met}) Native *E. coli* tRNA^{Met}. (C) Extent of translocation of fragmented tRNA^{Met} with single 2'-deoxy substitution at the indicated positions. Values were normalized with respect to translocation of the unsubstituted fragmented tRNA^{Met}, which was set to 100%. Results are the average of three experiments.

sugar conformation present in RNA (Saenger 1984). Therefore, it is not surprising that the fragmented tRNA containing the fully 2'-deoxyribose-substituted 5'-fragment does not fold properly and was translocated poorly. Interestingly, the fragmented tRNA containing the fully 2'-O-methyl-substituted 5'-fragment folds properly but was translocated poorly compared with the unsubstituted tRNA, indicating that one or more of the 2'-hydroxyl groups could be important for translocation (Fig. 2A). Although the 2'-O-methyl substitution permits the oxygen atom to accept a hydrogen bond, the inability to be a hydrogen bond donor may disrupt interactions with the ribosome, resulting in inhibition of translocation. Alternatively, the 2'-O-methyl group is bulky and the observed inhibition in translocation could be due to steric clash within the ribosome.

Identification of 2'-hydroxyl groups important for translocation

In order to determine the precise location of potential 2'-hydroxyl groups critical for translocation, we systematically tested single 2'-deoxy substitutions at positions 1-17. A toeprinting assay was used to monitor EF-G-dependent translocation by ribosomes containing a fragmented tRNA^{Met} in the P-site and an ASL4^{Phe} in the A-site. Surprisingly, single 2'-deoxy substitution at positions 1-17 within the P-site tRNA is readily tolerated by the ribosome, and no strong inhibition in translocation was detected in this single time-point experiment (Fig. 2B). However, subtle inhibition at positions 4, 6, and 12 (Fig. 2C) was observed, suggesting an involvement of these 2'-hydroxyl groups in translocation.

We next studied the kinetic behavior of the fragmented P-site-bound tRNA^{Met} incorporating 2'-deoxy substitutions at positions 1-17. Previous studies have shown that an ASL4^{Phe} in the ribosomal A-site will inhibit the rate of translocation up to 350-fold (Studer et al. 2003). Therefore, it is possible that an ASL4^{Phe} in the A-site may mask any subtle effects on translocation caused by the 2'-deoxy substitutions in the 5'-fragment. In order to avoid this potential problem, a full-length tRNA^{Phe} was used in the ribosomal A-site for the kinetic experiments. A full-length tRNA^{Phe} in the ribosomal A-site is translocated at a faster rate compared with the ASL; therefore, a reliable method to stop translocation at different time points is necessary to resolve the toeprinting assay.

It was demonstrated previously that the miscoding antibiotic neomycin is a potent inhibitor of EF-G-dependent translocation (Studer et al. 2003). We tested the ability of ribosomes containing tRNA^{fMet}, tRNA^{Met}, or a fragmented tRNA^{Met} in the P-site and a full-length tRNA^{Phe} transcript in the A-site to translocate in the presence of neomycin. Upon the addition of EF-G-GTP, no translocation was observed even after 30 min at 37°C, showing that neomycin could be used as an effective stop for translocation (Fig. 3A). Therefore, aliquots at specific time points during the translocation reaction were removed and added to extension mixes on ice containing reverse transcriptase and neomycin. At the completion of the time-course experiment, all samples were placed at 37°C for reverse transcriptase to synthesize the extension products.

Time-course toeprint assays using pretranslocation complex containing fragmented tRNA^{Met} with single 2'-deoxy substitutions in the P-site and a full-length tRNA^{Phe} in the A-site showed no significant difference in the rate of translocation (Fig. 3B). This indicates that ribose 2'-hydroxyl groups at positions 1-17 within the P-site tRNA are not essential for translocation. However, it is possible that 2'-deoxy substitution causes subtle inhibition of translocation that cannot be easily resolved using the toeprinting assay. We, therefore, analyzed the effect of 2'-deoxy substitutions

FIGURE 3. Time course of translocation of fragmented tRNA^{Met} with a single 2'-deoxy substitution in the small fragment. (A) Control time-course experiment showing inhibition of translocation by neomycin. Minus sign (–) indicates absence of EF-G. Neomycin was added to the pretranslocation complex, followed by the addition of EF-G-GTP. Aliquots of the reaction were taken at the indicated time points and analyzed by toeprinting. (B) Translocation of fragmented tRNA^{Met} incorporating single 2'-deoxy substitution at the indicated positions. EF-G-GTP was added to the pretranslocation complex, and aliquots of the reaction were taken at the indicated time points and mixed with extension reaction mix containing neomycin to stop translocation.

in the 5'-fragment of P-site tRNA using a more sensitive assay for translocation.

Translocation of fragmented tRNA monitored using a rapid kinetic assay

We recently described a fluorescence-based translocation assay that monitors the change in fluorescence of a pyrene molecule attached to the 3'-end of a short mRNA (Studer et al. 2003). As a first step, we formed pretranslocation complexes containing tRNA^{Met} or a tRNA^{Met} with a break in the D-loop in the P-site and an ASL^{Phe} in the A-site. EF-G was added to the pretranslocation complex by manual pipetting. A large decrease in the fluorescence is observed upon the addition of excess EF-G-GTP, which is indicative of translocation of the mRNA-tRNA complex (Studer et al. 2003). Consistent with the toeprinting results, these experiments also showed no difference in the rate of translocation with single 2'-deoxy substitutions at positions 1–17 (Table 1).

However, as mentioned above, the rate of translocation with an ASL in the ribosomal A-site is considerably slower compared with a peptidyl tRNA. Therefore, inhibition of translocation will only be observed if the magnitude of the effect due to the 2'-deoxy substitution is greater than the inhibition caused by the ASL. In order to overcome this potential problem, we used NAc-Phe-tRNA^{Phe}, an analog of peptidyl tRNA, in the ribosomal A-site and monitored the pre-steady-state kinetics of translocation with a stopped-flow instrument. Experiments were performed in a polyamine buffer that is optimal for translation (Bartetzko and Nierhaus 1988). Furthermore, the translocation assay was optimized using untagged EF-G and HPLC purified aminoacylated tRNA^{Phe}, which resulted in translocation rates similar to those reported by Wintermeyer and coworkers (Rodnina et al. 1997; Fig. 4A; Table 2).

Since the stopped-flow assay consumes a considerable amount of material, we chose to assay fragmented tRNAs containing single 2'-deoxy modifications at positions 1, 2, 3, 4, 5, 12, and 13. Positions 2, 3, 4, 5, 12, and 13 were selected because the X-ray crystal structure of the *Thermus thermophilus* 70S ribosome showed backbone contacts between the ribosome and these positions within the P- and the E-site tRNAs (Yusupov et al. 2001). Position 1 was chosen as a control because it does not form any contacts with the ribosome in the P- and E-sites. Pre-steady-state kinetic analysis showed that there was no difference in the rates of translocation between the fragmented tRNA^{Met} and any of the fragmented tRNA^{Met} containing single 2'-deoxy modifications (Fig. 4; Table 2), demonstrat-

TABLE 1. Translocation rates of 2'-deoxy-substituted tRNAs and an ASL^{Phe} from the ribosomal P- and A-sites, respectively

P-site substrate	k_{obs} (sec ⁻¹)
tRNA ^{Met}	0.005
Fragmented tRNA ^{Met}	0.003
2'-d1	0.003
2'-d2	0.003
2'-d3	0.003
2'-d4	0.006
2'-d5	0.004
2'-d6	0.003
2'-d7	0.003
2'-d8	0.005
2'-d9	0.004
2'-d10	0.005
2'-d11	0.005
2'-d12	0.004
2'-d13	0.005
2'-d14	0.004
2'-d15	0.004
2'-d16	0.004
2'-d17	0.005

Translocation rates were measured in conventional buffer with fivefold excess of C-terminal hexahistidine tagged EF-G at 25°C.

FIGURE 4. Rapid kinetic analysis of translocation. (A) Translocation kinetics of untagged EF-G (trace 1) and C-terminal hexahistidine tagged EF-G (trace 2). (B) Representative traces of single 2'-deoxy-substituted fragmented tRNA^{Met}. Trace 3 (red) indicates translocation of fragmented tRNA^{Met}; trace 4 (green), translocation of 2'-deoxy 1; trace 5 (black), translocation of 2'-deoxy 4. Fluorescence change is in arbitrary units.

ing that none of the tested ribose 2'-hydroxyl groups in the 5' strand of the acceptor arm of tRNA play a critical role during translocation from P- to E-site.

DISCUSSION

The ribosome forms a network of interactions with the tRNAs in the A-, the P-, and the E-sites (Yusupov et al. 2001). Some of these contacts between the tRNAs and the ribosome are likely to play a functional role during different steps of the translational cycle. Surprisingly, interactions between the acceptor arm of A-site tRNA and the 50S subunit are not critical for translocation. This was demonstrated by the translocation of an ASL4 from the ribosomal A- to the P-site (Joseph and Noller 1998). Since the ASL is composed of only 15 nt, it can be easily synthesized with various substitutions. This has facilitated the identification of functional groups within the ASL that are essential for A-site binding and translocation (Phelps et al. 2002; Phelps and Joseph 2005). It is important to note that even though an ASL is translocated from the A-site, the rate of translocation is at least 350-fold slower than a peptidyl-tRNA (Studer et al. 2003), suggesting that specific contacts between the acceptor arm of the tRNA and the ribosome or the three-dimensional shape of the A-site tRNA may accelerate the translocation of the mRNA-tRNA complex.

Interestingly, an ASL4 is not translocated from the ribosomal P-site, demonstrating the importance of the acceptor arm of P-site tRNA for translocation. The universally conserved bases C74 and C75 at the 3'-end of the tRNA form Watson-Crick base pairs with bases G2252 and G2251, respectively, in the P-loop of 23S rRNA, which are important for the peptidyl transferase reaction (Samaha et al. 1995; Nissen et al. 2000; Yusupov et al. 2001). In addition, the tRNA backbone (bk) at positions 3, 12, 13, and 76 in the acceptor arm of P-site tRNA contacts 23S rRNA at positions 2255–2256 bk, 1908–1909 bk, 1908–1909 bk, and 2585, respectively (Yusupov et al. 2001; Fig. 5). In the E-site tRNA, positions 2–71 bk, 3–5 bk, 71 bk, 73 bk, and 76 bk interact with 23S rRNA at positions 1850–1853 (Yusupov et al. 2001; Fig. 5). However, it is unclear which of these contacts are important for EF-G-dependent translocation. Previous studies, using a tRNA with a nick in the T-loop, showed that the 2'-hydroxyl groups at positions 71 and 76 in P-site tRNA are important for translocation (Feinberg and Joseph 2001). These 2'-hydroxyl groups contact 23S rRNA in the 50S E-site, establishing a functional role for these interactions in translocation. Thus, the fragmented tRNA approach is a powerful and cost-effective way for identifying functional groups within tRNA that are important for specific steps in translation.

Here we examined 2'-hydroxyl groups at positions 1–17 using a tRNA with a nick in the backbone at position 17/18. Our results showed that this fragmented tRNA bound efficiently to the ribosomal P-site and was translocated to the E-site. Single 2'-deoxy ribose-substituted 5'-small fragments were used to identify potential interactions that are important for translocation. Interestingly, our results show that interactions between the 2'-hydroxyl groups in the 5' strand of the acceptor arm of P-site tRNA (positions 1–17) and the ribosome are not essential for translocation. Therefore, it is possible that the three-dimensional shape of the tRNA in the

TABLE 2. Translocation rates of 2'-deoxy-substituted tRNAs

P-site substrate	A-site substrate	k_{obs} (sec ⁻¹)
tRNA ^{Met} a	fMet-Phe-tRNA ^{Phe} a	14.4 ± 0.2
tRNA ^{Met} a	fMet-Phe-tRNA ^{Phe} b	8.3 ± 0.1
tRNA ^{Met} b	NAC-Phe-tRNA ^{Phe} b	7.8 ± 0.4
Fragmented tRNA ^{Met}	NAC-Phe-tRNA ^{Phe} b	5.3 ± 0.2
2'-d1	NAC-Phe-tRNA ^{Phe} b	4.5 ± 0.2
2'-d2	NAC-Phe-tRNA ^{Phe} b	5.3 ± 0.6
2'-d3	NAC-Phe-tRNA ^{Phe} b	4.3 ± 0.2
2'-d4	NAC-Phe-tRNA ^{Phe} b	5.1 ± 0.2
2'-d5	NAC-Phe-tRNA ^{Phe} b	4.9 ± 0.7
2'-d12	NAC-Phe-tRNA ^{Phe} b	4.9 ± 0.2
2'-d13	NAC-Phe-tRNA ^{Phe} b	4.3 ± 0.8

Translocation rates were measured in polyamine buffer with five-fold excess of untagged EF-G at 25°C and the errors are ±SEM.

^aNative tRNA^{Met} and tRNA^{Phe}.

^bElongator tRNA^{Met} and tRNA^{Phe} transcripts.

FIGURE 5. Interaction of P- and E-site tRNAs with the 23S rRNA. The contacts observed in the 5.5 Å resolution structure of the 70S ribosome (Yusupov et al. 2001) are displayed using an all-atom model of the ribosome (Tung and Sanbonmatsu 2004). Nucleotides indicated in green and in blue are located in the P- and the E-sites, respectively. Backbone positions between 1–17 in P-tRNA (yellow) and E-tRNA (cyan) that interact with the 23S rRNA are indicated by red spheres. The figure was created using ViewerLite (Acclerys) and rendered with PovRay (<http://www.povray.org>).

P-site is more important for translocation than specific contacts with the 5' strand of the acceptor arm of the tRNA.

Chemical probing studies showed that following the peptidyl transferase reaction, the deacylated tRNA (P-site) and the peptidyl tRNA (A-site) move relative to the 50S subunit, forming hybrid P/E and A/P states, respectively (Moazed and Noller 1989). However, more recent studies indicate that the movement of deacylated tRNA from P/P to P/E state occurs after EF-G-GTP binds to the ribosome (Valle et al. 2003; Zavialov and Ehrenberg 2003). Specifically, a deacylated tRNA in the P-site stimulates binding of EF-G-GTP and GTP hydrolysis, while a peptidyl tRNA in the P-site inhibits these reactions (Zavialov and Ehrenberg 2003). The deacylated tRNA “unlocks” the 50S subunit and allows EF-G-GTP to induce the ratchet-like motion of the 30S subunit relative to the 50S subunit (Valle et al. 2003). This triggers movement of the deacylated tRNA from P/P to the P/E state and peptidyl tRNA from A/A to the A/P state. Hydrolysis of GTP by EF-G and the release of inorganic phosphate (Rodnina et al. 2001) then “unlocks” the 30S subunit, which triggers rotation of the 30S subunit head and its movement toward the 30S platform domain (Spahn et al. 2004; Schuwirth et al. 2005). The rotation of the 30S subunit head is thought to translocate the tRNA–mRNA complex relative to the 30S subunit (Spahn et al. 2004; Schuwirth et al. 2005). Thus, the ability of the deacylated tRNA in the P-site (in the P/P state) to move into the 50S subunit E-site (to the P/E state) profoundly affects EF-G-GTP binding, GTP-hydrolysis, and translocation. Consistent with this notion, sparsomycin, which can also induce translocation, is thought to stabilize the peptidyl-tRNA in A/P hybrid state (Fredrick and Noller 2003).

Movement of the acceptor arm of P-site tRNA into the E-site may establish specific contacts that are essential for unlocking the ribosome and for inducing GTP-hydrolysis. Interactions between the 2'-hydroxyl groups at positions 71 and 76 in the 3'-acceptor arm of P-site tRNA with the 23S rRNA are likely important for this step. However, none of the 2'-hydroxyl groups in the 5'-acceptor arm are important for translocation, suggesting that they may form nonspecific contacts with the ribosome that likely provide “leverage” for EF-G-induced ratcheting of the two subunits. Alternatively, it is possible that other functional groups, such as the nonbridging phosphate oxygen atoms, in the 5'-acceptor arm of P-site tRNA play an important role during translocation. Elucidating the interactions between the ribosome and its ligands that are important for specific steps in translation is essential for a mechanistic understanding of protein synthesis by the ribosome.

MATERIALS AND METHODS

Ribosomes, tRNA, and mRNA

Tight couple 70S ribosomes were isolated from *E. coli* MRE 600 cells, essentially as described (Powers and Noller 1991). Transfer RNA, native tRNA^{Met}, and tRNA^{Phe} were purchased from Sigma. *E. coli* elongator tRNA^{Met}, tRNA^{Phe}, and gene 32 mRNA were transcribed by T7 RNA polymerase from a linearized plasmid containing the appropriate insert. mRNA +9 was purchased from Dharmacon and labeled at the 3'-end with pyrene succinimide as described (Studer et al. 2003).

Synthesis and assembly of fragmented tRNA^{Met}

The large 3' tRNA^{Met} fragment corresponding to positions 18–76 was transcribed in vitro from a partially duplex synthetic DNA template using T7 RNA polymerase (Milligan and Uhlenbeck 1989). RNA transcripts were purified on a 10% denaturing polyacrylamide gel and recovered by passive elution, chloroform extraction, and ethanol precipitation. The smaller 5' tRNA^{Met} fragments corresponding to positions 1–18 were chemically synthesized on an ABI 392 DNA Synthesizer (Applied Biosystems) using an RNA phosphoramidite method. Site-specific incorporation of 2'-O-methyl and 2'-deoxy groups (Glen Research) was performed during solid-phase synthesis. The synthetic oligoribonucleotides were deprotected and purified on a 20% denaturing polyacrylamide gel. The 5' small fragment and the 3' large fragment were annealed essentially as described in Liu and Musier-Forsyth (1994). Briefly, 100 pmol of the 3' large fragment was mixed with 150 pmol of the 5' small fragment in 50 mM Hepes and heated for 3 min at 60°C. MgCl₂ was added to a final concentration of 10 mM, and the mixture was slowly cooled to room temperature and placed on ice. Samples were further prepared in appropriate translocation buffers prior to binding to the 70S–mRNA complex.

Nondenaturing polyacrylamide gel analysis

E. coli tRNA^{Met}, the 3' fragment, and the smaller 5' fragments were 5' end-labeled using [α -³²P]-ATP and T4 polynucleotide kinase

(New England Biolabs). The labeled RNAs were purified on denaturing polyacrylamide gels, and counts per minute were determined with a liquid scintillation counter (Beckman Coulter). The ^{32}P -labeled 5' fragment (50,000 cpm) was combined with 100 pmol of unlabeled 3' fragment and 150 pmol of the 5' large fragment and annealed as described above in 10 μL of final volume. The native tRNAs (50,000 cpm) also were folded by heating, addition of MgCl_2 to 10 mM, and slow cooling as above. Equal volumes of loading dye (40% glycerol, 0.2% bromophenol blue, 0.2% xylene cyanol; final concentration) were added, and 4 μL (10,000 cpm/lane) of sample was analyzed on a 1-mm nondenaturing 15% polyacrylamide (acrylamide/bisacrylamide 19:1) gel (40 mM Tris acetate at pH 7.5, 12 mM $\text{Mg}[\text{OAc}]_2$) (Fedor and Uhlenbeck 1990). The gels were run at 11W overnight in a 4°C cold room and visualized by autoradiography.

Native EF-G

Native *E. coli* EF-G lacking a histidine tag was purified via the IMPACT-CN method (New England Biolabs). Briefly, EF-G was cloned into the MCS of the pTYB1 vector directly adjacent to the self-cleaving intein tag, which also contains a chitin-binding domain. The plasmid was transformed into BL21-DE3 cells and grown at 37°C in LB medium containing 100 $\mu\text{g}/\text{mL}$ ampicillin until 0.5 OD_{600} was reached. The cultures were cooled to 15°C and induced with 0.3 mM IPTG and grown overnight. Cells were harvested at 5000 rpm in a Beckman JA-10 rotor at 4°C, washed, and resuspended in ~ 25 mL of a lysis buffer (20 mM Tris-HCl at pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100). Cells were lysed with a french press and the lysate was clarified by centrifugation at 20,000 rpm in a Beckman JA-17 rotor for 30 min at 4°C. A purification column was prepared by pouring ~ 15 mL of chitin beads per L of culture. The chitin column was equilibrated with ~ 10 volumes of column buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA) at 4°C. The clarified extract was loaded onto the column and then washed with ~ 300 mL of column buffer. EF-G is released from the column when the intein undergoes self-cleavage in the presence of thiols. Three bed volumes of column buffer containing 50 mM DTT was passed quickly through the column, the flow was stopped, and the column was stored at 4°C overnight. Following release from the intein tag, EF-G was eluted using column buffer; 1.5 mL fractions were collected and analyzed by SDS-PAGE. Fractions containing EF-G were pooled, concentrated using centricons (Millipore), and dialyzed against storage buffer (10 mM Tris-HCl at pH 8.0, 100 mM NaCl, 1 mM DTT, 50% Glycerol). Protein concentration was determined by Bradford assay, and activity was verified by toeprint and fluorescence assays.

Aminoacylation of tRNA

Aminoacylation of $\text{tRNA}_f^{\text{Met}}$ and tRNA^{Phe} was performed using purified *E. coli* histidine-tagged synthetase, essentially as described (Rodnina et al. 1994). Formylation of initiator $\text{tRNA}_f^{\text{Met}}$ was performed in parallel by adding N^{10} -formyltetrahydrofolic acid (0.3 mM final) and purified histidine-tagged *E. coli* methionyl tRNA formyltransferase to the $\text{tRNA}_f^{\text{Met}}$ aminoacylation reaction (Ramesh et al. 1997). Aminoacylation reactions were stopped by the addition of sodium acetate (pH 5.2) to 300 mM and placed on ice. Reactions were extracted twice with phenol and twice with

chloroform, and were precipitated using three volumes of ethanol. Samples were resuspended in 20 mM Tris acetate (pH 5.2) and were then purified by HPLC (Beckman Coulter) on a C18 reverse-phase Bondapak (Waters) column in 20 mM sodium acetate, 400 mM NaCl, and 10 mM MgCl_2 using a methanol gradient 0%–60% (Odom et al. 1988). Fractions were collected, ethanol-precipitated, and resuspended in 10 mM sodium acetate (pH 5.2). Aliquots were stored at -80°C . The extent of aminoacylation was verified by acid gel electrophoresis (Varshney et al. 1991), and the level of aminoacylation was $>95\%$. Concentrations were estimated by measuring the absorbance at 260 nm.

Aminoacylation and N-acetylation of tRNA^{Phe}

E. coli tRNA^{Phe} transcript (3000 pm) was annealed as described above and aminoacylated in 300 μL at 37°C for 20 min in charging reactions containing 25 mM Tris acetate (pH 7.5), 8 mM $\text{Mg}[\text{OAc}]_2$, 3 mM ATP, 100 mM NH_4Cl , 30 mM KCl, 1 mM DTT, 20 μM phenylalanine, and tRNA-free *E. coli* tRNA phenylalanine specific synthetase. Aminoacylation reactions were stopped by the addition of NaAc to 300 mM and placed on ice. Aminoacylated tRNA^{Phe} was then N-acetylated using the acetic-anhydride method (Haenni and Chapeville 1966). Reactions were extracted twice with phenol and twice with chloroform, divided into two tubes (~ 250 μL each), and precipitated using three volumes of ethanol. Samples were resuspended and pooled in 20 mM Tris acetate (pH 5.2) prior to HPLC purification as described as above.

Formation of translocation complexes and toeprint analysis

Pretranslocation complexes were formed using ribosomes programmed with gene 32 mRNA and containing tRNA^{Met} in the P-site and ASL4^{Phe} or *E. coli* tRNA^{Phe} in the A-site, as described in Joseph and Noller (1998). The final concentration of the components was 0.4 μM tight-couple ribosomes, 0.6 μM gene 32 mRNA, 2 μM tRNA^{Met} (or annealed-fragmented tRNA^{Met}), 6 μM ASL4^{Phe} , or 0.8 μM *E. coli* tRNA^{Phe} in conventional buffer (80 mM K-cacodylate at pH 7.2, 20 mM $\text{Mg}[\text{OAc}]_2$, 150 mM NH_4Cl at final concentration). Translocation reactions were performed by the addition of 2.0 μM EF-G and 300 μM GTP (final concentrations) to the complexes. Aliquots of the reaction mixture were taken at defined time points and placed in an extension mix on ice containing the antibiotic neomycin (100 μM). Zero time point lacked EF-G-GTP. All samples (single time point and time course) were placed in a water bath at 37°C for 10 min for extension of the primer by reverse transcriptase. Extension products were resolved on a 10% denaturing polyacrylamide gel, and the gels were quantified with a Molecular Dynamics PhosphorImager. Rectangles were used to quantify the toeprint bands corresponding to pre- and post-translocation for each reaction time using the volume integrate function of IMAGEQUANT software (Molecular Dynamics). Total counts for each time point are the sums of pre- and post-translocation bands. The extent of translocation was calculated by dividing the post-translocation counts by the total counts for each reaction. The translocation of modified tRNAs was normalized with respect to translocation of unmodified fragmented tRNA^{Met} from each experiment (set at 100% for the 30-min time point).

Fluorescence assay

Pretranslocation complexes were formed essentially as described in Studer et al. (2003). Ribosomes (0.25 μ M) were incubated for 10 min at 42°C for in a polyamine buffer (20 mM Hepes-KOH at pH 7.6, 6 mM MgCl₂, 150 mM NH₄Cl, 4 mM β -mercaptoethanol, 0.05 mM spermine, 2 mM spermidine) (Bartetzko and Nierhaus 1988), followed by incubation for 10 min at 37°C. Pyrene-modified mRNA +9 (0.3 μ M) was added to the ribosome and the complexes were incubated at 37°C for 10 min. Next, tRNA^{Met} (1.25 μ M) or fragmented tRNA^{Met} (1.25 μ M, substituted or unsubstituted) was added, and the complexes were incubated at 37°C for 20 min followed by addition of ASL4^{Phe} (3.75 μ M) and incubation at 37°C for 20 min and placed on ice. GTP was added to a final concentration of 1 mM. The EF-G-GTP mix was formed by incubating EF-G (1.25 μ M) and GTP (1 mM) in buffer for 10 min at 37°C.

Time-course fluorescence measurements

Time-course fluorescence emission spectrums of translocation complexes were analyzed on a photon-counting instrument (FluoroMax-3, J.Y. Horiba Inc.). The temperature of the sample was maintained constant at 25°C by connecting the instrument to an external, circulating water bath. A 160- μ L aliquot of pretranslocation complex was transferred to a square Sub-Micro cell (Starna Cell 45 mm $\times$ 12.5 mm $\times$ 12.5 mm). Using a time-based scan, the sample was excited at 343-nm wavelength (excitation band pass, 1 nm), and the emission at 376-nm wavelength (emission band pass, 1 nm; path length, 3 mm) was acquired over time. Translocation was initiated by manual addition of 5 μ L of the EF-G-GTP mix. A delay of <10 sec occurs prior to data collection, and data were collected every 10 sec. The data were fit by least squares fitting to the single exponential equation

$$Y = Y_{\max}[1 - \exp(-kx)]$$

where $Y_{\max}$ is the maximum extent of translocation, Y is the fraction translocated at time x , and k is the observed rate constant (GRAPHPAD PRISM; GraphPad). The observed end points for each reaction were used to fit the data. The rates were derived from the average of at least three independent experiments and are within the 95% confidence intervals. Errors in the rate were <20%.

Stopped-flow fluorescence measurements

Rapid kinetic experiments were performed at 25°C on a stopped-flow instrument (μ SFM-20, BioLogic). A FC-08 cuvette with a light path of 0.8 mm was used with a dead time of 1.6 msec. The complexes were prepared essentially as described above and mixed at 1:1 ratio (mixing volume of 120 μ L). The excitation wavelength was 343 nm (band pass, 10 nm), and the fluorescence emission was measured after passing a long-pass filter 361 AELP (Omega Optical) installed in front of the detector.

In all cases, one syringe contained pretranslocation complexes, and the other syringe contained fivefold excess EF-G-GTP mix. Each experiment typically consists of at least six shots. Each shot is analyzed separately and then averaged to determine the rate of translocation per experiment. Shot-to-shot variance was <10%; from experiment to experiment, less than twofold. Translocation

data were analyzed by least squares fitting to the single exponential equation $Y = (\text{Span} \cdot e^{-(k_{\text{app}} \cdot \text{time})}) + \text{Plateau}$, using GraphPad Prism software.

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