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Messenger RNA interactions in the decoding center control the rate of translocation

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During protein synthesis, mRNA and tRNAs are iteratively translocated by the ribosome. Precisely what molecular event is rate limiting for translocation is not known. Here we show that disruption of the interactions between the A-site codon and the ribosome accelerates translocation, suggesting that the release of the mRNA from the decoding center of the ribosome is the rate-limiting step of translocation. These results provide insight into the molecular mechanism of translocation.

Ribosomes are responsible for protein synthesis in all organisms. A central step in the elongation cycle of protein synthesis is the translocation of the tRNAs from the aminoacyl site (A-site) to the peptidyl site (P-site) and to the exit site (E-site). The associated mRNA also moves by one codon in this process. Pre–steady-state kinetic analysis of translocation showed that a structural rearrangement in the ribosome is the rate-limiting step for translocation[1]. However, it is not known what precisely is the structural rearrangement that controls the rate of translocation. Structural studies revealed that domain IV of EF-G is located in the A-site of the 30S subunit (decoding center)[2–5]. On the basis of this observation, it was proposed that during translocation EF-G might disrupt the interactions made by the codon-anticodon helix with the decoding center[3,5].

To determine whether the interactions between mRNA and the decoding center are important for translocation, we made modifications to the 2′-hydroxyl groups of the A-site codon. All three 2′-hydroxyl groups of the A-site codon form hydrogen bonds with the universally conserved 16S rRNA bases G530, A1492 or A1493 (**Fig. 1a**)[6]. To abolish the hydrogen bonds between the mRNA and the decoding center, we synthesized mRNAs with single 2′-deoxynucleotide substitutions in the A-site codon (positions +4, +5 and +6) (**Fig. 1b**). Because the 2′-deoxynucleotide substitutions in the A-site codon may destabilize the binding of the A-site peptidyl-tRNA[7,8], we determined the dissociation rate constant (k_{off}) of fMet-Phe-tRNA^{Phe} from the A-site by filter binding (**Fig. 1c**). The k_{off} of [^{35}S]fMet-Phe-tRNA^{Phe} with the control mRNA was 0.04 ± 0.01 min^{-1}, whereas single 2′-deoxynucleotide substitutions in the A-site codon increased the k_{off} by two- to seven-fold (mRNA+4D = 0.07 ± 0.03 min^{-1}, mRNA+5D = 0.27 ± 0.06 min^{-1} and mRNA+6D = 0.19 ± 0.03 min^{-1}). A similar trend was also observed when a tripeptide formation assay was used to measure the k_{off} (**Supplementary Fig. 1**). We also measured the k_{off} of *N*-acetyl-Phe-tRNA^{Phe}, an analog of peptidyl-tRNA, from the A-site (**Fig. 1d**). We again observed that mRNA+5D and mRNA+6D increased the k_{off} of *N*-acetyl-Phe-tRNA^{Phe} from the A-site (**Supplementary Table 1**). These results show that single 2′-deoxynucleotide substitution at positions +5 or +6 in the mRNA severely destabilizes the binding of the peptidyl tRNA in the A-site.

To determine the rate of translocation, we used a fluorescence-based, pre–steady-state kinetic assay[9]. The pre-translocation complex, containing a deacylated tRNA^{Met} in the P-site and an *N*-acetyl-Phe-tRNA^{Phe} in the A-site, was rapidly mixed with EF-G–GTP to monitor the time course of translocation (**Supplementary Discussion**). The kinetics of mRNA translocation showed two phases, with apparent rate

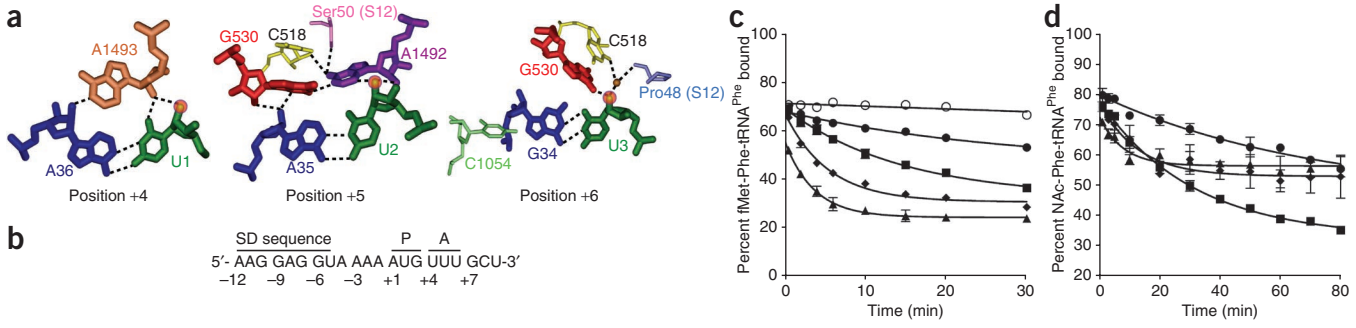

Figure 1 Interactions in the decoding center and the kinetics of tRNA dissociation. (**a**) Interaction of the A-site codon with the decoding center. Shown are mRNA bases (dark green), tRNA anticodon bases (blue), 16S rRNA bases and ribosomal proteins (in other colors). The orange spheres highlight the 2′-hydroxyl groups of the mRNA at positions +4 to +6. (**b**) Sequence of the mRNA used in this study. The Shine-Dalgarno (SD) sequence and P- and A-site codons are labeled. (**c**) Dissociation kinetics of [^{35}S]fMet-Phe-tRNA^{Phe} from the A-site. Data shown are for control mRNA (●), mRNA+4D (■), mRNA+5D (▲) and mRNA+6D (◆). The dissociation kinetics of [^{35}S]fMet-tRNA^{fMet} from the P site is also shown as a control (○). (**d**) Dissociation kinetics of *N*-acetyl-[^{14}C]Phe-tRNA^{Phe} from the A-site. Symbols are described as in (**c**). Error bars represent s.d. from three experiments.

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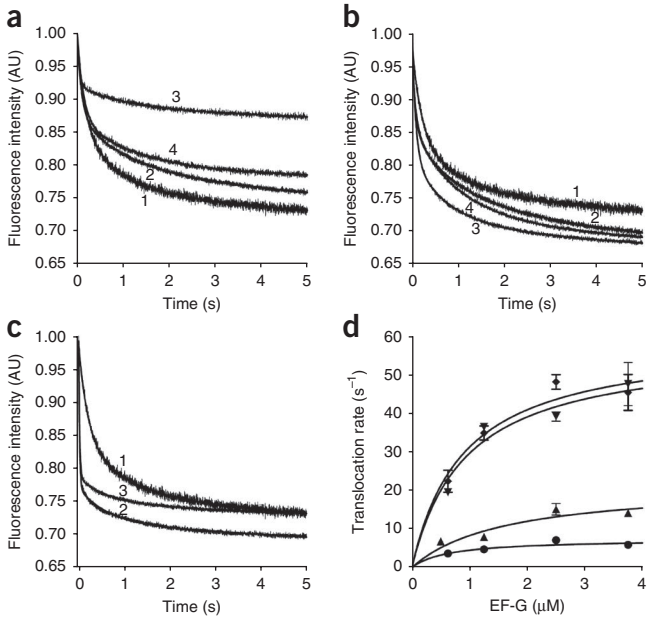

Figure 2 Translocation kinetics of 2′-deoxynucleotide– and 2′-fluoro–substituted mRNAs. (**a**) Translocation kinetics of control mRNA (trace 1), mRNA+4D (trace 2), mRNA+5D (trace 3) and mRNA+6D (trace 4). The maximum fluorescence intensity was normalized to 1 and is shown in arbitrary units (AU). (**b**) Translocation kinetics of control mRNA (trace 1), mRNA+4F (trace 2), mRNA+5F (trace 3) and mRNA+6F (trace 4). (**c**) Translocation kinetics of control mRNA (trace 1), mRNA+5F+6F (trace 2) and mRNA+4F+5F+6F (trace 3). (**d**) Plot of the translocation rates (k_{av}) at increasing concentrations of EF-G. Data shown are for control mRNA (●), mRNA+5F (▲), mRNA+5F+6F (▼) and mRNA+4F+5F+6F (◆). Error bars represent s.d. from three experiments.

constants k_1 and k_2 for the fast and slow phases, respectively (**Fig. 2a**). The biphasic kinetics of mRNA translocation is of unknown cause but has been reported previously[10,11]. We calculated the weighted average rate constant (k_{av}) as described recently[11]. The control mRNA translocated at an average rate of 4.7 ± 1.2 s^{-1} (**Supplementary Table 2**). We consistently observed a two-fold higher rate of translocation for mRNA+5D (k_{av} = 10.5 ± 2.8 s^{-1}) compared to the control mRNA. The rates of translocation of mRNA+4D or mRNA+6D were similar to those for the control mRNA. Compared to the control mRNA, the overall change in fluorescence intensity due to translocation was lower with mRNA+4D, mRNA+5D and mRNA+6D. This may be due to the lower occupancy of the A-site by *N*-acetyl-Phe-tRNA^{Phe} with the 2′-deoxynucleotide–substituted mRNAs. To verify that mRNAs with single 2′-deoxynucleotide substitutions in the A-site codon are translocated by the ribosome, we used a tripeptide formation assay (**Supplementary Methods**). The rates of tripeptide formation with mRNA+4D, mRNA+5D and mRNA+6D were similar to those for the control mRNA, showing that translocation is not inhibited by the single 2′-deoxynucleotide substitutions in the A-site codon (**Supplementary Fig. 2**).

To see whether the modest stimulation in translocation with the single 2′-deoxynucleotide substitution is specific to position +5, we tested mRNAs with single 2′-deoxynucleotide substitution at positions −3, −2, −1, +1, +2, +3, +7, +8 and +9 (**Fig. 1b**). The translocation rates of mRNAs with these substitutions were similar to those for the control mRNA (**Supplementary Fig. 3** and **Supplementary Table 3**). Thus, the increase in the rate of translocation is specific to mRNA+5D.

Because the 2′-fluoro substitution is less disruptive than the 2′-deoxynucleotide substitution[12], we synthesized mRNAs having single 2′-fluoro substitutions at positions +4, +5 or +6. The k_{off} of *N*-acetyl-Phe-tRNA^{Phe} from the A-site was one-quarter to one-half that seen with the single 2′-fluoro–substituted mRNAs compared to mRNAs having the single 2′-deoxynucleotide substitutions (**Supplementary Table 1**, **Supplementary Figs. 4** and **5**).

We next determined the rate of translocation of the 2′-fluoro–substituted mRNAs (**Fig. 2b**). Single 2′-fluoro substitution at position +5 or +6 increased the rate of translocation by ~2-fold (**Supplementary Table 2**). Our results suggest that the loss of hydrogen bonds between the 2′-hydroxyl groups at positions +5 or +6 and the decoding center stimulates translocation. We wondered whether multiple 2′-fluoro substitutions in the A-site codon would increase the rate of translocation even more. To test this idea, we synthesized an mRNA with two 2′-fluoro substitutions at positions +5 and +6 (mRNA+5F+6F) and another mRNA with three 2′-fluoro substitutions at positions +4, +5 and +6 (mRNA+4F+5F+6F). The k_{off} of *N*-acetyl-Phe-tRNA^{Phe} from the A-site was higher with the double and triple 2′-fluoro–substituted mRNAs compared to the single 2′-fluoro–substituted mRNAs (0.063 ± 0.005 min^{-1} and 0.176 ± 0.029 min^{-1} for mRNA+5F+6F and mRNA+4F+5F+6F, respectively) (**Supplementary Fig. 4** and **Supplementary Table 1**). Strikingly, the rate of translocation was increased by seven-fold with the double and triple 2′-fluoro–substituted mRNAs (k_{av} = 35–38 s^{-1}) compared to the control mRNA (**Fig. 2c**) (**Supplementary Table 2**).

To determine the apparent affinity of EF-G for the pre-translocation complex ($K_{1/2}$) and the maximal rate of translocation (k_{trans}), we measured translocation rates at several concentrations of EF-G. The $K_{1/2}$ values did not differ significantly, indicating that EF-G binding is not affected by the 2′-fluoro substitutions in the mRNA ($K_{1/2}$ = 0.6 μM, 1.6 μM, 0.9 μM and 0.9 μM for control mRNA, mRNA+5F, mRNA+5F+6F and mRNA+4F+5F+6F, respectively) (**Fig. 2d**). The k_{trans} for control mRNA, mRNA+5F, mRNA+5F+6F and mRNA+4F+5F+6F were 7 s^{-1}, 22 s^{-1}, 58 s^{-1} and 60 s^{-1}, respectively (**Fig. 2d**). These results indicate that disruption of the interactions between the A-site codon and the decoding center accelerates translocation.

A recent study showed that treatment of ribosomes with colicin E3 accelerates translocation[13]. Colicin E3 cleaves the 16S rRNA between bases A1493 and G1494, and this was thought to increase the conformational flexibility of the decoding center. The stability

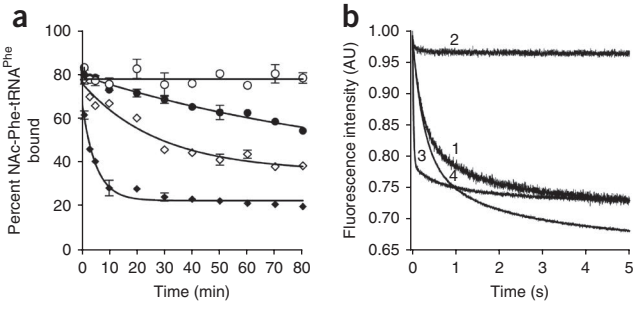

Figure 3 Inhibition of tRNA dissociation and translocation by viomycin. (**a**) Dissociation kinetics of *N*-acetyl-[^{14}C]Phe-tRNA^{Phe} from the A-site in the presence of viomycin. Shown are data for control mRNA without (●) and with viomycin (○) and mRNA+4F+5F+6F without (◆) and with viomycin (◇). Error bars represent s.d. from three experiments. (**b**) Inhibition of translocation by viomycin. Shown are translocation of the control mRNA without (trace 1) and with viomycin (trace 2) and translocation of mRNA+4F+5F+6F without (trace 3) and with viomycin (trace 4).

of the peptidyl-tRNA in the A-site, however, was not reduced after colicin E3 treatment[13]. By contrast, the single, double and triple 2′-fluoro–substituted mRNAs destabilized the binding of the peptidyl tRNA in the A site by 3-, 5- and 15-fold, respectively.

Destabilizing the binding of the peptidyl tRNA in the A-site may lower the free energy of activation for translocation (ground state destabilization) and thus lead to the higher translocation rate[14]. To investigate this further, we used the antibiotic viomycin. Viomycin interacts with A1492 and A1493 in the decoding center[15] and increases the affinity of peptidyl-tRNA to the A site[16]. Viomycin inhibits translocation by stabilizing the peptidyl tRNA in the A/A[17,18] or in the A/P state[19]. In the presence of viomycin, the dissociation of *N*-acetyl-Phe-tRNA^{Phe} was extremely slow with the control mRNA, so that we could not reliably measure the k_{off} (**Fig. 3a**). With the triple fluoro mRNA, viomycin decreased the k_{off} of *N*-acetyl-Phe-tRNA^{Phe} from the A-site by a factor of 5 (0.036 ± 0.002 min^{-1}) compared to in the absence of the drug. We next determined the rate of translocation with the control and the triple 2′-fluoro mRNAs in the presence of viomycin. Viomycin completely inhibited translocation of the control mRNA, whereas the translocation rate of the triple 2′-fluoro mRNA was decreased by a factor of 18 ($k_{av} = 2.1 \pm 0.1$ s^{-1}) (**Fig. 3b** and **Supplementary Fig. 6**). These results indicate that viomycin inhibits the translocation of the 2′-fluoro mRNA by partially restoring the stability of the peptidyl-tRNA in the A-site.

Translocation is a complex process consisting of extensive conformational changes in the ribosome. The rate-limiting step for mRNA-tRNA movement during translocation is thought to be a conformational change in the 30S subunit[1,20]. Despite many studies, the fundamental question of what precisely occurs during the rate-limiting step of translocation remains unresolved. On the basis of our findings, we propose that the rate-limiting step of translocation corresponds to the disruption of the interactions between the A-site codon and bases G530, A1492 and A1493 in the decoding center. Disruption of the interactions in the decoding center would stimulate the swiveling of the head domain and the partial unratcheting of the body and platform domains of the 30S subunit[4]. Translocation is then completed by back swiveling of the head domain and complete unratcheting of the 30S subunit[4,11]. In addition, our results suggest that faster translocation is achieved at the expense of loss of stability of the A-site tRNA. Thus, ribosomes are finely tuned machines that balance the need for stably binding a tRNA in the A-site with the need for rapid translocation.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

P.K.K. and S.J. designed the experiments, P.K.K. performed the experiments, and P.K.K. and S.J. discussed the results and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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