Table S1. Testing the Effect on –1PRF of the aa-tRNAs Decoding mRNA Downstream of the Frameshifting Site, Related to Figure 1

| Ternary complex concentration, μM | | Rate, $s^{-1}$ [a] | | –1PRF, % [b] |
|---|---|---|---|---|
| Val | Phe | Val, –1-frame | Phe, 0-frame | |
| 1.5 | 1.5 | 0.3 ± 0.05 | 0.09 ± 0.01 | 75 ± 10 |
| 0 | 1.5 | – | 0.07 ± 0.02 | – |
| 1.5 | 0 | 0.2 ± 0.05 | – | – |
| 1 | 4 | 0.5 ± 0.06 | 0.06 ± 0.02 | 80 ± 10 |
| 4 | 1 | 0.3 ± 0.05 | 0.05 ± 0.01 | 80 ± 10 |

[a] Rates of incorporation of Val and Phe into peptide, as determined by global fitting of kinetic data similar to those shown in Figure 1D-H.

[b] Average –1PRF efficiency calculated from end levels of Val and Phe incorporation.

Table S2. Testing the Functionality of Fluorescence-Labeled Ribosome Complexes, Related to Figures 2 and 4

| Step | Rates, $s^{-1}$ | | |
|---|---|---|---|
| | Wild-type[c] | L12/EF-G[d] | S13/$tRNA^{Leu}$ [e] |
| Tyr[a] | 9.9 ± 0.5 | 9.5 ± 0.4 | 15.8 ± 0.6 |
| $Tl_{MY}$[b] | 2.7 ± 0.1 | 2.0 ± 0.1 | 3.1 ± 0.1 |
| Leu[a] | 13.6 ± 3.2 | 14.9 ± 2.5 | 9.8 ± 1.2 |
| Lys[a] | 5.1 ± 0.4 | 6.0 ± 0.7 | 5.7 ± 0.3 |
| Val[a] | 0.2 ± 0.05 | 0.5 ± 0.1 | 0.5 ± 0.03 |
| Phe[a] | 5.3 ± 0.6 | 4.0 ± 0.5 | 3.3 ± 0.2 |

[a] Rates of incorporation of various amino acids into peptide on –/– mRNA.

[b] Rate of the 1st translocation event ($Tl_{MY}$; translocation of MY-$tRNA^{Tyr}$ from the A to P site).

[c] Unmodified ribosomes and tRNAs.

[d] Ribosomes reconstituted with L12(Alx) and EF-G(QSY).

[e] Ribosome complexes contained S13(AttoQ) and $tRNA^{Leu}$(Flu).

Table S3. Fitting of Fluorescence Changes upon MYLK-tRNA$^{Lys}$ Translocation by Numerical Integration, Related to Figures 2 and 4

| Observables | Rate, $s^{-1}$ | | | |
|---|---|---|---|---|
| | –/– mRNA, +K | –/– mRNA, +K/V/F | +/+ mRNA, +K | +/+ mRNA, +K/V/F |
| FRET L12–EF-G | | | | |
| $k_{Lys}$ | 4.8[a] | | 4.8[a] | |
| $k_{EFG1}$ (EF-G binding) | >10[b] | | 4.8 ± 0.1 | |
| $k_{EFG2}$ (EF-G release) | 3.0 ± 0.2 | | 0.2± 0.01 | |
| $\Delta F$ of tRNA$^{Leu}$ | | | | |
| $k_{Lys}$ | 4.9[a] | 4.9[a] | 4.9[a] | 4.9[a] |
| $k_{app1}$ | –[c] | –[c] | >10[b,d] | >10[b,d] |
| $k_{app2}$ | 9.0 ± 0.2[e] | 9.8 ± 0.6[e] | 0.9 ± 0.1[f] | 0.9 ± 0.1[f] |
| $k_{app3}$ | 3.0 ± 0.2[g] | 3.3 ± 0.1[g] | | 0.2 ± 0.02[g] |
| FRET S13–tRNA$^{Leu}$ | | | | |
| $k_{Lys}$ | 5.7[a] | 5.7[a] | 5.7[a] | 5.7[a] |
| $k_{app1}$ | 9.0 | 9.0 | 0.9 | 0.9 |
| $k_{app2}$ | 3.4 ± 0.2[g] | 3.3 ± 0.3[g] | 0.25 ± 0.03[g] | 0.21 ± 0.1[g] |

Apparent rate constants were determined by numerical integration using the following reaction sequences:

$POST_{Leu} \xrightarrow{k_{Lys}} PRE_{Lys} \xrightarrow{k_{EFG\,1}} PRE_{EFG} \xrightarrow{k_{EFG2}} POST + EF-G$, for binding and release of EF-G, which corresponded to the minimum number of steps required for fitting, and

$POST_{Leu} \xrightarrow{k_{Lys}} PRE_{Lys} \xrightarrow{k_{EFG\,1}} PRE_{EFG} \xrightarrow{k_{app1}} POST1 \xrightarrow{k_{app2}} POST2 \xrightarrow{k_{app3}} POST3$, for tRNA translocation with the minimum number of $k_{app}$ terms required for fitting, two or three as appropriate. Fitting to a smaller number of steps did not yield satisfactory fits.

[a]The values for $k_{Lys}$ were determined by quench flow (Table S3) and used as a fixed parameter for fitting.

[b] As EF-G binding precedes translocation, EF-G recruitment step was included in fitting the FRET changes for +/+ mRNA (4.8 $s^{-1}$, taken as a fixed parameter in fitting). For the –/– mRNA this was not necessary, because EF-G recruitment was relatively rapid.

[c]–, not observed.

[d] The lowest estimate compatible with the fit; the step represents the transition from $PRE_{EF\text{-}G}$ to POST1 (see main text).

[e] Represent translocation from $PRE_{EF\text{-}G}$ to POST1/2 on –/– mRNA; the rate corresponding to $k_{app1}$ observed for the +/+ mRNA was not resolved, as the rates, >10 $s^{-1}$ and 9-10 $s^{-1}$, were too similar. The steps are distinguished based on the differences in the fluorescence change of $tRNA^{Leu}$ (Fig. 4H and I, see also Discussion).

[f] Transition POST1 to POST2 for +/+ mRNAs; represents the sum of the rate constants at the kinetic partitioning branch (Figure 5).

[g] Transition from POST2 to POST3, which entails dissociation of EF-G, $tRNA^{Leu}$, and the backward rotation of the 30S head.