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Supplementary Information for

Translation initiation site of mRNA is selected through dynamic interaction with the ribosome

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This PDF file includes:

Supplementary Information Text

Methods

Plasmids for protein expression

The plasmids used for overexpression of *E. coli* initiation factors were originally obtained from Dr. Harry Noller of University of California, Santa Cruz. IF1 was wild type. IF2 and IF3 contained a His6 tag fused to the C terminus.

Purification of the 30S and 50S ribosomal subunits

E. coli strains MRE600 (BCRC#11862) and KLF203 (originally obtained from Dr. Harry Noller of University of California, Santa Cruz) were used for ribosome purification. In KLF203 (1), the genomic sequence encoding ribosomal protein bS16 was fused at its C-terminus with the biotinylation domain of the biotin carboxyl carrier protein (BCCP). Cells were grown at 37°C in LB (Luria-Bertani) broth to an OD600 of ~ 0.6, pelleted by centrifuging at 4°C, and stored at -80°C. The following purification procedures, modified from those reported in the literature (2, 3), were carried out at 4°C or on ice. The frozen cell pellet was resuspended in Buffer A [20 mM Tris-HCl, pH 7.2, 100 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol (β-ME), 0.5 mM EDTA, 20 U/ml RNase inhibitors (SMOBIO #RI1000), and a tablet of protease inhibitors (Thermo Scientific #A32965)] with a ratio of 10 ml buffer per gram of cells and lysed by three to four passes through French press (Constant Systems, CF Range) at 20 kpsi. The cell lysate was cleared twice by centrifugation at 13,000 rpm for 30 min each (using Megafuge 16R with the Fiberlite F15-6x100y rotor, ThermoFisher). The supernatant was collected and carefully layered atop an equal volume of 37.7% sucrose cushion prepared in Buffer B (20 mM Tris-HCl, pH 7.2, 500 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM β-ME, and 0.5 mM EDTA). The sample was subjected to centrifugation at 33,800 rpm (using CP80WX with the P40ST rotor, Hitachi) for 17 h to pellet down the 70S ribosomes. To further isolate the individual ribosomal subunits, the pellets were gently washed with Buffer D [10 mM Tris-acetate, pH 7.5, 60 mM NH_4Cl, 1 mM $Mg(OAc)_2$, 6 mM β-ME, and 0.5 mM EDTA], dried, and resuspended in 500 μl Buffer D per tube to separate the ribosomes into the 50S and 30S subunits. The sample was layered atop 10–40% sucrose gradient prepared in Buffer D and centrifuged at 21,100 rpm (using CP80WX with the P28S rotor, Hitachi) for 17 h. The 50S and 30S subunits were separately collected and dialyzed against Buffer C [20 mM Tris-acetate, pH 7.5, 60 mM NH_4Cl, 7.5 mM $Mg(OAc)_2$, 6 mM β-ME, and 0.5 mM EDTA]. The samples were aliquoted, flash frozen with liquid nitrogen, and stored at -80°C.

Purification of initiation factors, IF1, IF2 and IF3

To facilitate purification of IF1, a His6 tag followed by TEV cutting site (4) was inserted into the N-terminus of IF1. BL21-CodonPlus (DE3)-RIPL competent cells (Agilent #230280) were transformed with the reconstructed IF1 plasmid and grown at 37°C in LB broth containing 50 μg/ml kanamycin to an OD600

of ~ 0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce protein expression. Cells were grown for another 2 h at 37°C, pelleted by centrifuging at 4°C, and stored at -80°C. The frozen cell pellet (~ 2 g) was resuspended in 20 ml PB buffer (50 mM Tris-HCl, pH 7.5, 60 mM NH_4Cl, 7 mM $MgCl_2$, 6 mM β-ME, 15% glycerol) supplemented with 15 mM imidazole and protease inhibitor (Thermo #A32965)] and disrupted by three passes through French press at 20 kpsi. The cell lysate was then centrifuged 3 times at 13,000 rpm for 30 min. The supernatant was loaded to the HisTrap FF Crude 5 ml column (GE #17528601). The column was washed with 50 ml PB buffer supplemented with 500 mM KCl and 15 mM imidazole. IF1 protein was then eluted by a gradient of 15-250 mM imidazole in PB buffer. The eluted sample was dialyzed against proTEV buffer [50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM Dithiothreitol (DTT) and 15% glycerol]. The His6 tag was cut by the ProTEV Plus protease (Promega #V6101) at 30°C for 2 h. The ProTEV digested IF1 was co-incubated with the His Mag beads (GE #11530894) in PB buffer containing 15 mM imidazole. The unbound IF1 was collected and dialyzed against SP buffer (25 mM Tris-HCl, pH 7.5, 60 mM NH_4Cl, 7 mM $MgCl_2$, 6 mM β-ME and 15% glycerol) and loaded to the HiTrap SP HP 5 ml column. The column was washed with 50 ml SP buffer. IF1 protein was eluted with a gradient of 60-2000 mM NH_4Cl in SP buffer and dialyzed against PB buffer. Samples were frozen by liquid nitrogen and stored at -80°C. IF2 and IF3 were purified in a similar procedure, except that the ProTEV digestion and the rebinding to the His Mag beads were omitted.

Preparation of RNA constructs for optical tweezers experiments

DNA sequences of interest were chemically synthesized and inserted into the pT7SP6 plasmid (5) at the NdeI and BsrGI restriction sites. The plasmid was originally derived from pVE60hp and preserved the ribosomal binding site and other features suitable for optical tweezers measurements with bound ribosomes (6). The constructed plasmids were linearized by BssSαI to prepare templates for run-off transcription. RNAs were synthesized using the MEGAscript T7 *in vitro* transcription kit (Ambion #AM1333) and purified by the MEGAclear kit (Ambion #AM1908). The RNAs (~ 1750 nt long) were annealed to two complementary DNA handles with lengths of 735 bp at the 5' end and 919 bp at the 3' end, respectively, leaving the flanked region with the ribosomal binding site and indicated hairpin-forming sequences. DNA handles were prepared by PCR. The 3' handle was biotin-labeled directly through a biotinylated primer. The 5' handle was PCR-amplified first, followed by end-labeling with digoxigenin using T4 DNA polymerase and digoxigenin-11-dUTP (Roche #11093088910).

Optical tweezers experiments

To measure the unfolding force and size of RNA structures on optical tweezers, one tether of the RNA constructs was specifically held between two surface-modified polystyrene beads (~2 μm in diameter) through the tagged DNA handles. One bead (Spherotech #SVP-20-5) was coated with streptavidin for biotin binding and the other (Spherotech #CP-20-10) was carboxyl-modified and cross-linked with anti-

digoxigenin antibodies. The RNA was attached to the beads as previously described (5). Briefly, the streptavidin-coated bead was fixed on the tip of a micropipette and the antibody-coated bead was trapped by laser beams. We repeatedly pulled the molecules by moving the laser trap at a constant speed (100 nm/s), such that the force was gradually increased from 2 and 30 pN (to unfold the structures) and then in a reverse direction from 30 to 2 pN (to let the structures refold). The experiment was done in TLB-t buffer [40 mM HEPES-KOH, pH 7.5, 70 mM NH_4Cl, 7 mM $Mg(OAc)_2$, 1 mM DTT].

For the preparation of 30S PIC, 6 μl of TLB-t buffer containing 0.2 μM of the indicated mRNA construct, 0.8 μM of 30S subunits, and 10 U of RNase inhibitors were incubated at 37°C for 30 min. Initiator tRNA (1.5 μM) was included when 30S IC was made. For 70S $IC^{+IF1/2/3}$, the reaction mixture was 15 μl and contained 0.2 μM of the indicated mRNA construct, 1.6 μM 70S ribosomes, 2.5 μM IF1, 2.5 μM IF3, 5 μM IF2, 1.6 μM fMet-$tRNA^{fMet}$, 1 mM GTP, and 10 U of RNase inhibitors. The reaction was performed at 37°C for 20 min. The formed RNA-ribosome complexes were diluted with 10 volumes of TLB-t buffer and then attached to the beads for optical tweezers measurements as described above.

Plasmid construction for smFRET experiments

Compared with optical tweezers, measurements in smFRET need shorter flanking handles for the attachment of dye pairs. In this report, the 5' handle and 3' handle were 18 bp and 26 bp, respectively. We inserted the chemically synthesized DNA sequences into pVE60hp between the KpnI and BamHI sites; the KpnI site is only a few base pairs downstream of the T7 promoter. When linearizing the newly constructed plasmids at the BamHI site, run-off transcription from the T7 promoter would generate suitable RNA lengths (~ 100 nt) for annealing of complementary DNA handles at both ends. Two unique restriction sites (XmaI and BsrGI) were included in the inserted sequence to facilitate future cloning of related constructs. RNAs were in vitro transcribed and purified as described above. In some plasmids, we inserted the SP6 promoter between the KpnI and XmaI sites and transcribed the RNA using the MEGAscript SP6 *in vitro* transcription kit (Ambion #AM1330).

smFRET experiments

To increase the handle annealing efficiency and minimize RNA dimerization (likely through the hairpin-forming sequences), we have optimized the annealing protocol as follows. The RNA, the 5' handle [single-stranded DNA (ssDNA) with a biotin tag and a Cy5 dye labeled at the 3' end and 5' end, respectively], and the 3' handle (ssDNA with a Cy3 dye at the 3' end) were mixed with a ratio of 0.5/0.25/0.5 μM in 40 mM HEPES-KOH, pH7.5. The mixture was heated at 92°C for 2 min (denaturing), instantly put on ice for 10 min (minimizing dimerization), and then incubated at 45°C for 15 min (handle annealing). The annealed constructs were diluted to 50 pM (the RNA concentration) in TLB buffer [40 mM HEPES-KOH, pH 7.5, 70 mM NH_4Cl, 7 mM $Mg(OAc)_2$, 6 mM β-ME] and injected into the channels divided by double-sided tapes in a slide chamber. The slide chamber was passivated by polyethylene glycol (PEG) and biotin-conjugated

PEG in a ratio of 50:1, such that the biotinylated RNA constructs could be immobilized through sandwiched NeutrAvidin proteins (7). The slide was assembled on a home-built total internal reflection fluorescence (TIRF) microscopic system for smFRET measurements, as described previously (5). Image recording was set at 50 ms/frame for 60 s under illumination of the 532-nm green laser (75 mW, CL532-075-L, CrystaLaser). During the last second of recording, the green laser was turned off and the 638-nm red laser (35 mW, DL638-035, CrystaLaser) was turned on to check the co-localization of an acceptor dye (Cy5). This was helpful to exclude the molecules that contained only the donor dye (Cy3). Such recording was repeated at least 10 times from different visual fields within the same reaction channel. The measurements were done at ambient temperature (~25°C, in an isolated, air-conditioned room) in imaging buffer: TLB buffer supplemented with 2.67 mM Trolox, 2.5 mM protocatechuic acid (PCA), and 0.21 unit/ml protocatechuate-3,4-dioxygenase (PCD).

To study the ribosome-induced conformational change of the RNA constructs, a mixture containing the indicated components (see below) was prepared in imaging buffer and injected into the chamber, which was then incubated at 37°C for 15 min and returned to ~25°C before recording. The injected mixture contained 0.1 μM 30S subunits and, whenever specified, 0.1 μM $tRNA^{fMet}$ (or fMet-$tRNA^{fMet}$) and/or 0.5 μM each of IF1, IF2, and IF3. For 70S $IC^{+IF1/2/3}$, the mixture contained 0.1 μM 30S subunits, 0.1 μM 50S subunits, 0.1 μM fMet-$tRNA^{fMet}$ and 0.5 μM each of IF1, IF2, and IF3. The imaging buffer consisted of TLB buffer [40 mM HEPES-KOH, pH 7.5, 70 mM NH_4Cl, 7 mM $Mg(OAc)_2$, 6 mM β-ME], 2.67 mM Trolox, 2.5 mM protocatechuic acid (PCA), and 0.21 unit/ml protocatechuate-3,4-dioxygenase (PCD). To remove free and weakly bound components, the chamber was washed sequentially with TLB and imaging buffers, 70 μl each. To minimize the operational inconsistency by hand, we used a programmable electronic pipette (Thermo Scientific #46200400) to control the buffer flow with a consistent rate (14 μl/s). After washing, the same chamber was recorded again and the recording time was set for 2 s (instead of 60 s). The shortened recording time would minimize the variation of FRET distribution, as different 30S complexes would continue dissociation to different extent (e.g., see Fig. 7A and 7D).

Estimation of errors

A bootstrapping method was used to estimate the errors of rates from model-fitted FRET time traces. This method was modified from a previous report (8): (i) all dwell times acquired under the same conditions were pooled; (ii) the same number of dwell times were randomly resampled and averaged, and a rate was calculated by the reciprocal of the average; (iii) the previous step was repeated for 1,000 times; and (iv) the rate and error were determined as the mean and standard deviation of the 1,000 estimates.

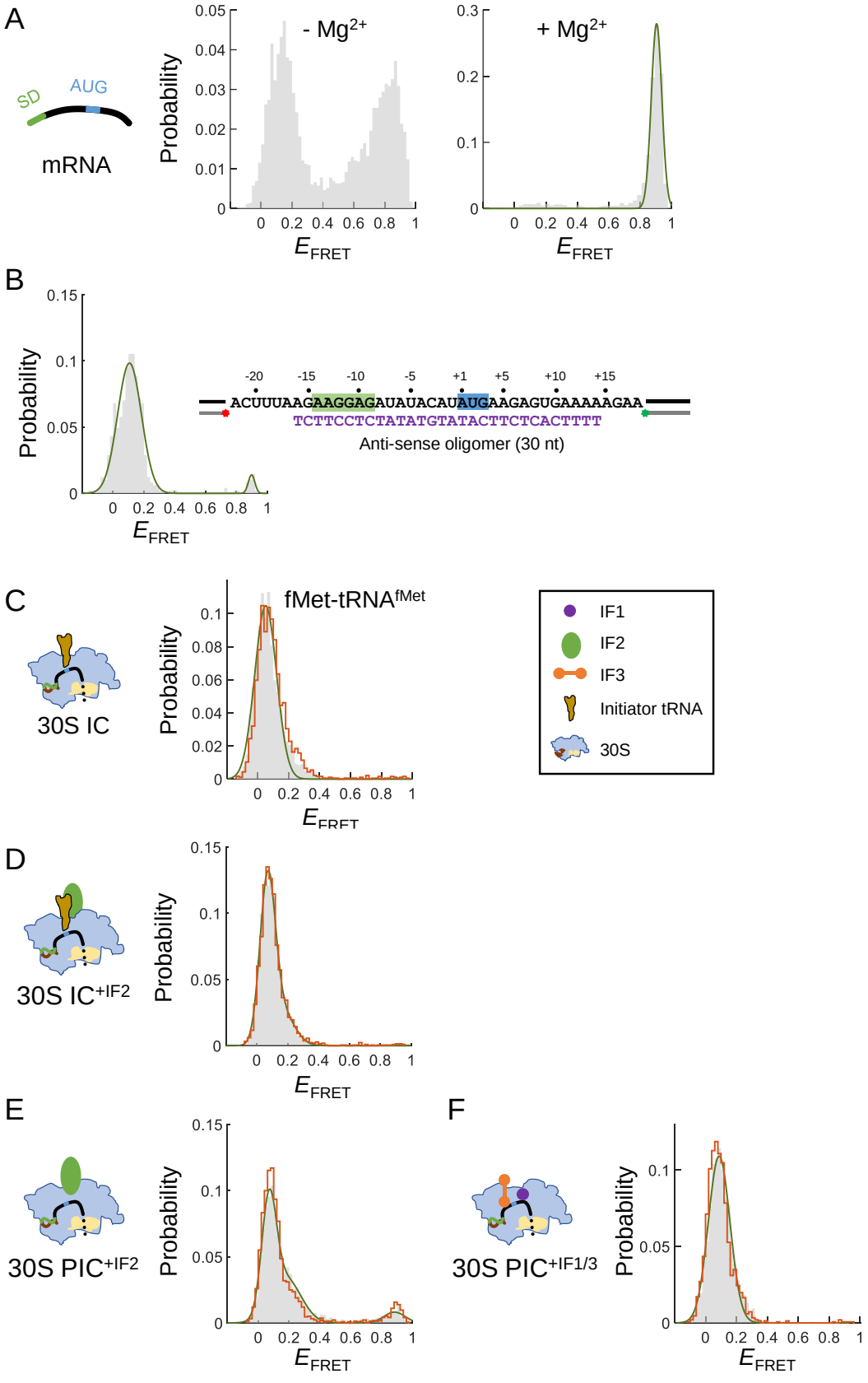
A
SD
AUG
mRNA
- Mg2+
+ Mg2+
Probability
EFRET
B
-20 -15 -10 -5 +1 +5 +10 +15
ACUUUAAGAAGGAGAUAUACAUAUGAAGAGUGAAAAAGAA
TCTTCCTCTATATGTATACTTCTCACTTTT
Anti-sense oligomer (30 nt)
C
30S IC
fMet-tRNAfMet
IF1
IF2
IF3
Initiator tRNA
30S
D
30S IC+IF2
E
30S PIC+IF2
F
30S PIC+IF1/3

Fig. S1. Control experiments with F+18. (**A**) E_{FRET} histograms of F+18 in the absence (left) and presence (right) of 7 mM magnesium acetate. The E_{FRET} distributions indicate that F+18 existed in heterogeneous conformations in the absence of Mg^{2+}, but those conformations were converted to more compact structures with Mg^{2+}, reflecting a sharp peak at high E_{FRET}. (**B**) E_{FRET} histograms of F+18 annealed with an anti-sense DNA oligomer that is complementary to the ribosome binding site. (**C**–**F**) E_{FRET} histograms of F+18 in the states of 30S IC with fMet-$tRNA^{fMet}$ (C), 30S IC^{+IF2} (D), 30S PIC^{+IF2} (E), and 30S $PIC^{+IF1/3}$ (F). Except for 30S PIC^{+IF2}, these E_{FRET} distributions were almost indistinguishable to that of 30S IC with $tRNA^{fMet}$ (uncharged; Fig. 2C), indicating that the formation of these types of 30S IC/PIC was almost complete and stable. Though the 30S PIC^{+IF2} (E) shows a minor population at the high E_{FRET}, the FRET distribution was essentially unchanged after buffer washing, suggesting that this complex also had comparable stability. Gray bars and orange lines represent histograms before and after buffer washing, respectively. Green curves are Gaussian functions fitted to the gray bars.

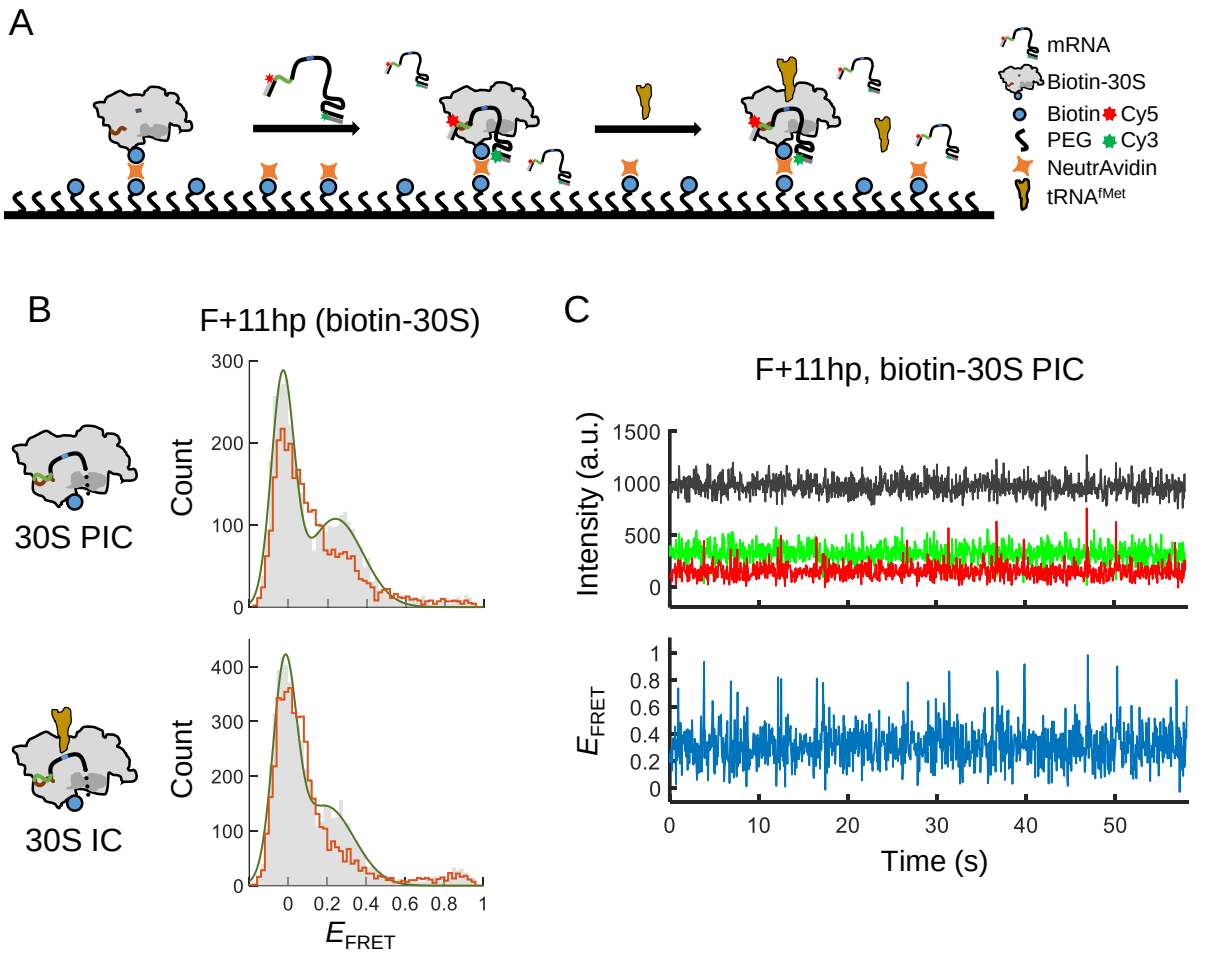

Fig. S2. Dynamic conformational change of mRNA is not resulted from repeated disassociation-association of 30S subunits. (**A**) Experimental setup of smFRET with surface-immobilized 30S subunits. The 30S subunit contained a biotin tag for immobilization. Dye-pair-labeled mRNA (50 pM), with or without initiator tRNA, was injected into the chamber for the formation of initiation complexes. (**B**) E_{FRET} histograms of F+11hp in the states of 30S PIC (top) and 30S IC (bottom). Gray bars and orange lines represent histograms (in count) before and after buffer washing, respectively. Green curves are Gaussian functions fitted to the gray bars. Note that, after buffer washing, a large fraction of the mRNA remained on the immobilized 30S subunits (> 80%), whereas only 21.7 ± 5.7% of the complexes were retained when immobilizing the mRNA (see Fig. 4B). This was probably because, compared with the bulky 30S subunit (~ 850 kDa), the mRNA construct (47.5 kDa) experienced less drag force and thus could survive under the buffer stream. (**C**) A representative time trace for F+11hp in the 30S PIC state. The top panel shows the intensity changes of Cy3 (green), Cy5 (red), and the sum of both dyes (black; shifted by 500 a.u. for clarity). The corresponding E_{FRET} change is shown below. While the FRET signal was fluctuating over time, the sum of intensity remained stable, indicating that the dye-pair-labeled mRNA was persistently associated with the immobilized 30S subunit. a.u., arbitrary unit.

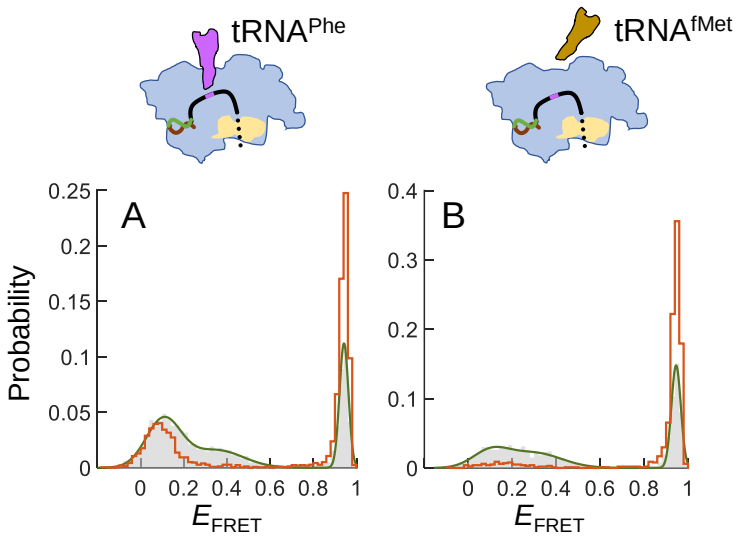

Fig. S3. Cognate codon-anticodon interactions in the P site stabilized 30S IC with structured mRNA. E_{FRET} histograms of Fuuu+11hp in the presence of 30S subunits and the cognate $tRNA^{Phe}$ (**A**) or non-cognate $tRNA^{fMet}$ (**B**). Fuuu+11hp was the same as F+11hp, except that the UUU codon was substituted for the AUG start codon. Gray bars and orange lines represent histograms before and after buffer washing, respectively. Green curves are Gaussian functions fitted to the gray bars.

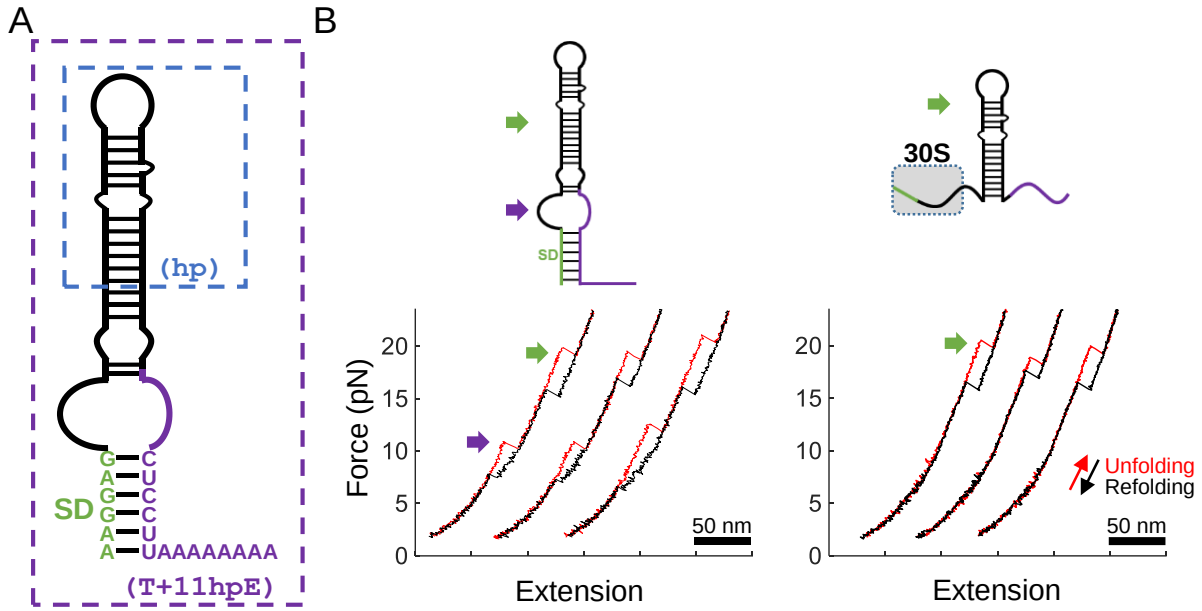

Fig. S4. The binding of the 30S subunit to the mRNA hairpin can be distinguished when another helix is formed with the SD sequence. (**A**) A schematic drawing shows the predicted secondary structure of the T+11hpE downstream hairpin. The hairpin was derived from T+11hp by extending 3 bp at the bottom of the hairpin and inserting a sequence (purple) after the 3' end to form a short helix with the upstream SD sequence (green). (**B**) Representative force-extension curves for T+11hpE in the states of mRNA alone (left) and 30S PIC or 30S IC (right). When pulled on optical tweezers, the mRNA construct by itself showed two transitions, unzipping of the SD helix (purple arrow) at a lower force followed by unfolding of the hp hairpin plus the 3 bp extension (green arrow) at a higher force (left). When the 30S PIC (or 30S IC) was correctly formed, the 30S subunit would bind to the SD sequence and disrupt the helix, and thus only the hpE unfolding signal was detectable (right).

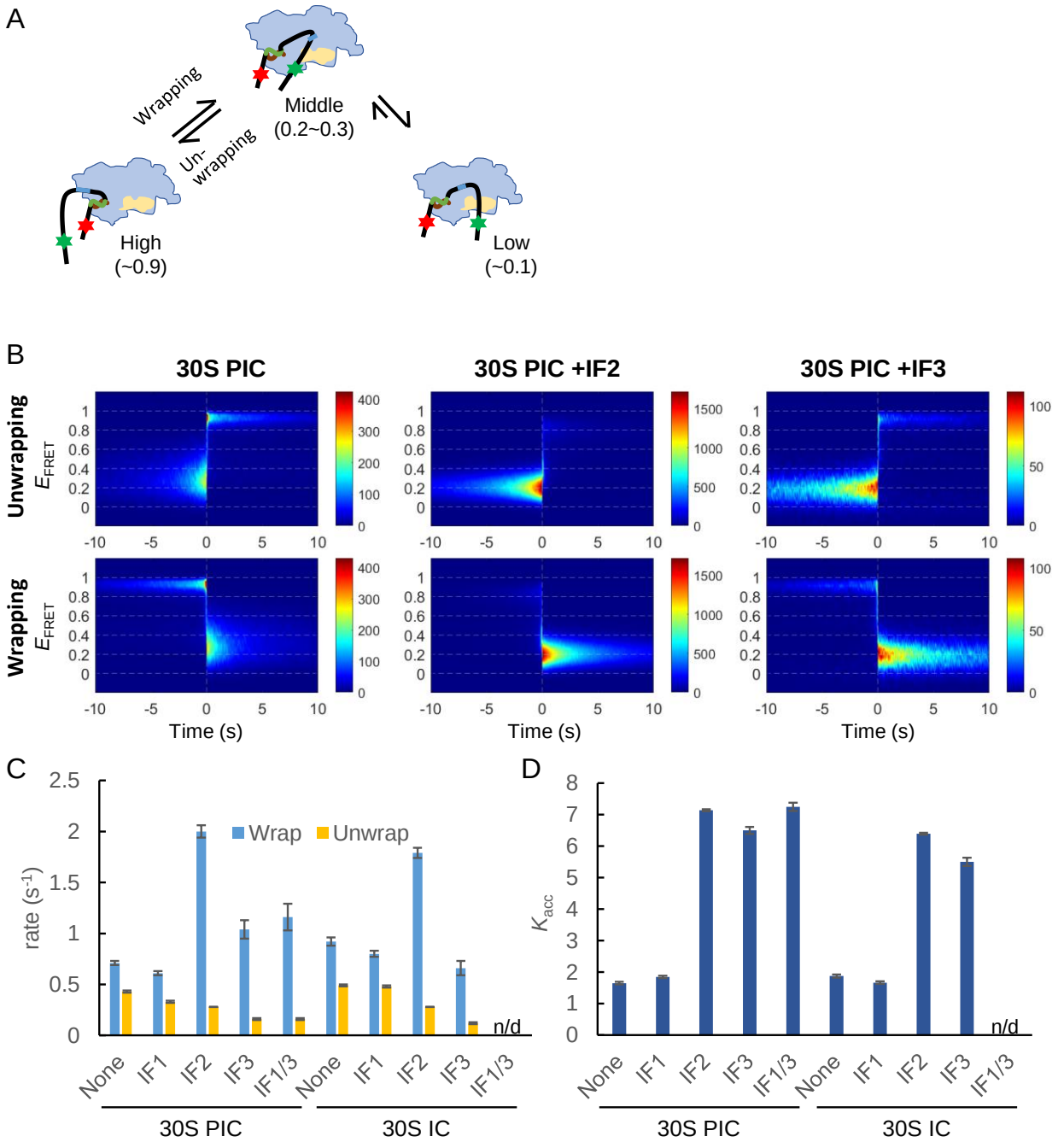

Fig. S5. Kinetics of the structured F+8 mRNA accommodation onto the 30S subunit. (**A**) A schematic drawing (see Fig. 2G) shows a possible conformational change of mRNA in the 30S PIC. The corresponding FRET states are indicated. (**B**) Contour plots of post-synchronization for the transitions between the middle (E_{FRET} 0.2~0.3) and the high (E_{FRET} ~0.9) FRET states of 30S PIC with F+8. Data were processed from model-fitted FRET time traces. The initiation factor added to the reaction is indicated on top. (**C**) Rate constants of the high-to-middle FRET transition ($k_{wrapping}$) and the middle-to-high FRET transition ($k_{unwrapping}$) of 30S PIC and 30S IC with F+8. Initiation factors added to the reaction are indicated. The rates and errors

were estimated by a bootstrapping method (see SI Methods). (**D**) Equilibrium constant (K_{acc}) of the accommodation (high-to-middle) reaction, determined by the ratio of $k_{wrapping}/k_{unwrapping}$ from (C). n/d, not determined.

Table S1. Percentages and major E_{FRET} peaks of mRNA in the ribosome-bound states under various conditions.

Construct		30S PIC		30S IC		70S IC	
		Incubation	Washed	Incubation	Washed	Incubation	Washed
F+18	Bound (%)	98.1±1.1	82.0±8.7	99.6±0.3	99.1±0.2	99.2±1.3	98.9±0.6
	E_{FRET} peak	0.07/0.10	0.07/0.22	0.08	0.06	0.08	0.06
F+18 with IF2	Bound (%)	90.0	90.3	98.8	98.5		
	E_{FRET} peak	0.07/0.18	0.08/0.22	0.07/0.17	0.07/0.14		
F+18 with IF1/3	Bound (%)	99.4	99.3				
	E_{FRET} peak	0.09	0.08				
FwSD+18	Bound (%)	4.6	1.2	52.6	46.7		
	E_{FRET} peak	n/d	n/d	0.08/0.15	0.11		
FnSD+18	Bound (%)	~0	~0	~0	~0		
	E_{FRET} peak	n/d	n/d	n/d	n/d		
FwSD+18 with IF1/3	Bound (%)	46.2	34.3				
	E_{FRET} peak	0.04	0.03				
FnSD+18 with IF1/3	Bound (%)	~0	~0				
	E_{FRET} peak	n/d	n/d				
F+14	Bound (%)	89.5±2.9	57.3±23.3	96.4±1.7	95.3±2.5		
	E_{FRET} peak	0.07/0.19	0.11/0.26	0.05/0.20	0.07/0.24		
F+11	Bound (%)	72.1±8.5	16.1±8.2	89.8±5.4	87.5±8.9		
	E_{FRET} peak	0.29/0.08	0.28	0.07/0.24	0.08/0.23		
F+8	Bound (%)	60.1±1.3	13.3±3.5	86.3±7.0	83.3±1.8		
	E_{FRET} peak	~0.22	~0.18	0.10	0.09		
F+8 with IF1	Bound (%)	61.1±3.3	6.6±5.1	83.1±1.1	76.1±1.7		
	E_{FRET} peak	~0.22	~0.18	0.11	0.10		
F+8 with IF2	Bound (%)	94.3	65.8	94.3	61.7		
	E_{FRET} peak	0.24	0.24	0.23/0.32	0.24		
F+8 with IF3	Bound (%)	95.9±1.0	78.9±2.5	97.1±0.7	77.0±3.2		
	E_{FRET} peak	0.24/0.11	0.24/0.09	0.11, 0.23	0.25/0.15		
F+8 with IF1/3	Bound (%)	96.6±2.9	77.2±4.3	97.7±0.7	77.3±5.9		
	E_{FRET} peak	0.19/0.05	0.24/0.09	0.16	0.22		
F+11hp	Bound (%)	65.6±11.1	21.7±5.7	73.8±14.3	62.0±10.3	99.6±0.5	61.7±3.8
	E_{FRET} peak	0.23/0.05	~0.20	0.09/0.20	0.11	0.11	0.15
F+11hp with IF1	Bound (%)	66.9±3.8	18.5±6.0	77.0±15.8	65.8±17.3		
	E_{FRET} peak	0.21/0.06	~0.20	0.09/0.27	0.10		
F+11hp with IF2	Bound (%)	89.2	22.4	92.6	54.2		
	E_{FRET} peak	0.33/0.16	~0.26	0.08/0.29	~0.23		

F+11hp with IF3	Bound (%)	95.2±0.2	73.3±6.1	94.8±2.2	79.9±6.4
	E_{FRET} peak	0.17	0.17	0.04, 0.18	0.17
F+11hp with IF1/3	Bound (%)	97.2±0.8	80.2±4.8	97.1±0.7	81.8±6.4
	E_{FRET} peak	0.12	0.17	0.11	0.17

Construct		**30S IC (with $tRNA^{Phe}$)**		**30S IC (with $tRNA^{fMet}$)**	
		Incubation	Washed	Incubation	Washed
Fuuu+11hp	Bound (%)	60.6	22.4	47.3	~0
	E_{FRET} peak	0.11/0.35	0.09	0.10/0.30	n/d

For each mRNA construct in the indicated states, the sample was measured before ("Incubation") and after ("Washed") buffer washing. The percentage of mRNA in the ribosome-bound states (usually below E_{FRET} 0.5) was calibrated by the distribution with mRNA alone and is shown as mean±S.D. (with at least three independent experiments) or mean (with two independent experiments). The data from those independent experiments were also pooled to determine the major E_{FRET} peak of the ribosome-bound states by Gaussian fitting to the histogram. When relevant, two peaks were determined and shown (a slash to separate the major peak (front) and the minor peak (back); a comma to separate two roughly equivalent peaks). The peak was not determined (n/d) when the population was too low or too diverse to fit.

Table S2. Unfolding forces and sizes of the mRNA hairpins in various ribosome-bound states.

Construct		mRNA	30S PIC	30S IC	70S IC	
T+11hp	Force (pN)	19.1±1.1	18.6±1.1	14.5±0.9	13.9±1.0	
	Size (nt)	30.6±2.1	30.2±1.7	24.2±2.0	24.4±2.3	
	Trace no.	1665	987	347	1081	
	Molecule no.	26	20	13 (8)	22 (7)	
T+11hpM	Force (pN)	22.9±0.8	22.8±0.8	20.6±1.2	20.9±1.1	
	Size (nt)	29.0±1.3	28.6±1.6	23.8±1.5	24.3±1.8	
	Trace no.	1177	713	937	2103	
	Molecule no.	21	12	18 (7)	34 (5)	
T+10hpM	Force (pN)	22.4±0.8		19.5±0.9	19.9±1.1	
	Size (nt)	29.1±1.7		23.6±2.2	22.9±1.9	
	Trace no.	677		380	337	
	Molecule no.	17		12 (73)	8 (41)	
T+9hpM	Force (pN)	22.3±0.7		n/a		
	Size (nt)	29.0±1.5		n/a		
	Trace no.	357		n/a		
	Molecule no.	7		0 (23)		
T+8hpM	Force (pN)	22.4±0.8		n/a		
	Size (nt)	29.4±1.6		n/a		
	Trace no.	848		n/a		
	Molecule no.	20		0 (44)		**30S PIC$^{+IF1/3}$**
T+11hpE	Force (pN)	21.1±1.1	19.9±1.3	19.0±1.1		20.0±0.9
	Size (nt)	37.0±1.9	36.1±2.7	30.5±2.6		35.7±2.8
	Trace no.	1161	1408	241		834
	Molecule no.	23	25 (13)	5 (33)		62

For each mRNA construct in the indicated states, the number of molecules measured on optical tweezers and the total number of traces (force-extension curves) obtained from the molecules are indicated. Data are shown as mean±S.D. Because the formation efficiency of 30S IC and 70S IC (in which the hairpin should be partially opened) varied for each construct, a molecule may show a structural transition indistinguishable from that of the free mRNA, indicating that the molecule was not successfully forming the initiation complex. In this case, the results from these molecules were not counted, and the number of such molecules is shown in parentheses. n/a, not available.

Table S3. Disassembly kinetics of 30S IC and 30S PIC.

	30S IC with F+11hp		30S IC with F+8		30S PIC^{+IF3} with F+8	30S IC with F+18	
	Buffer wash	IF3 wash	Buffer wash	IF3 wash	Buffer wash	Buffer wash	IF3 wash
A_1	0.92	0.74	0.91	0.71	0.58	0.97	0.98
k_1 (s^{-1})	$7.7\pm1.6 \times10^{-5}$	$2.0\pm0.05 \times10^{-4}$	$3.5\pm0.4 \times10^{-5}$	$1.8\pm0.1 \times10^{-4}$	$1.5\pm1.4 \times10^{-4}$	$1.8\pm0.5 \times10^{-6}$	$2.1\pm0.2 \times10^{-5}$
A_2	0.08	0.26	0.09	0.29	0.42	0.03	0.02
k_2 (s^{-1})	$7.3\pm8.8 \times10^{-3}$	$2.4\pm0.2 \times10^{-3}$	$1.5\pm0.4 \times10^{-2}$	$5.6\pm1.0 \times10^{-3}$	$1.9\pm1.7 \times10^{-3}$	$2.1\pm0.3 \times10^{-2}$	$4.3\pm4.7 \times10^{-2}$
R^2	0.976	0.999	0.994	0.999	0.987	0.996	0.995

Data were from fitting the following equation to each data set in Fig. 7A and 7D: $p = A_1*\exp(-k_1*t) + A_2*\exp(-k_2*t)$, where p is the probability, k_1 and k_2 are the rate constants, t is time, A_1 and A_2 are the proportions with $A_1 + A_2 = 1$.

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