Supporting Information

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SI Methods

Synthetic Aptamer Preparation. Modified RNA strands and a DNA splint were purchased from Dharmacon and IDT, respectively. To prepare the aptamer of the TPP riboswitch, two modified RNA strands [5′-biotin-GGAACCAAACGACUCGGGGUGCCCUU-CUG-C(Dy647)-GUGAAGGCUGAGAAAUACCCGUA-3′ and 5′-phosphate-UCACCUGAUCUGGA-U(Dy547)-AAUGCCAGC-GUAGGGAAGUCA-3′] were annealed with a DNA splint (5′-CATTATCCAGATCAGGTGATACGGGTATTTCTCAGC-3′) by slowly cooling from 94 °C to 4 °C in annealing buffer [10 mM Tris·HCl (pH 8.0) with 50 mM NaCl] and ligated with T4 RNA ligase 2 (New England Biolabs) (1). Ligation products were purified using denaturing 15% PAGE with 8 M urea and folded by slow cooling from 80 °C to 4 °C in annealing buffer.

EC Reconstitution. The Dy647-labeled seed RNA and template and nontemplate DNA strands were purchased from Dharmacon and IDT, respectively (Table S1). The seed RNA (800 nM) was incubated in a tube with a template DNA strand (200 nM) and T7 RNAP (40 nM; New England Biolabs) for 50 min at 37 °C. A donor-labeled UTP (Cy3-UTP, 100 μM; GE Healthcare) and a nontemplate DNA strand (400 nM) were added to the tube and incubated for 20 min before the single-molecule experiment. The Cy3 is attached at the C5 position of the nucleobase.

Single-Molecule FRET Experiments. Polymer-coated quartz slides were prepared using a 1:40 mixture of biotin-PEG (biotin-PEG-5000; Laysan Bio, Inc.) and PEG (m-PEG-5000; Laysan Bio, Inc.) (2). After streptavidin (0.2 mg/mL; Invitrogen) coating of the slides for 5 min, the EC (400 pM) was immobilized on the polymer-coated surface via streptavidin–biotin interaction and imaged using a homemade wide-field total internal reflection fluorescence microscope equipped with a green laser (532 nm, Compass215M; Coherent) and a red laser (640 nm, Cube640-100C; Coherent). An electron multiplying charge-coupled device camera (Ixon DV897; Andor Technology) was used as an imaging device. All experiments were performed at 37 °C with 50-ms exposure times in an alternating laser excitation (ALEX) mode (3, 4). Because the experiments were performed in an ALEX mode, the actual time resolution of the experiments was 100 ms. For the elongation experiments, NTP was injected with reaction buffer [10 mM Tris·HCl (pH 8.0), 20 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM protocatechuic acid, 500 nM protocatechuate 3,4-dioxygenase]. IDL (7.0; ITT), MATLAB (R2010a; The MathWorks), and Origin (8.0; OriginLab) were used for data analysis. Various NTP and TPP concentrations were used for transcription elongation, but high FRET fractions for the RNA transcripts were determined in the presence of 200 μM TPP.

The Promoter-Initiated Pause Assay with *Escherichia coli* and T7 RNAP. Linear DNA template (50 nM) containing the T7A1 promoter followed by the TPP riboswitch were incubated with 50 nM *E. coli* RNAP holoenzyme for 10 min at 37 °C in transcription buffer [20 mM Tris·HCl (pH 8.0), 20 mM NaCl, 14 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA]. Halted ECs (+20) were formed by adding 150 μM ApU dinucleotide (Trilink), 20 μM GTP and CTP, 1 μM ATP, and 10 μCi [α-^{32}P]ATP (3,000 Ci/mmol). Transcription was then allowed to resume by addition of all four NTPs (50 μM) and heparin (100 μg/mL). Samples were removed at the indicated times and mixed with 2× loading dye [7 M urea, 90 mM Tris·borate (pH 8.3), 2.5 mM Na_2EDTA, 0.02% bromophenol blue, 0.02% xylene cyanol]. For the chase lane, ECs were further incubated with 500 μM NTPs for 10 min at 37 °C before being quenched. RNA products were separated on a 6% denaturing polyacrylamide gel. In vitro transcription assays using T7 RNAP were performed similarly, except for the inclusion of a DNA template with the T7 promoter instead of the T7A1 promoter and forming the halted ECs (+20) in the absence of ApU. The initial transcribed sequences (+1 to +21) are ATCGAGAGGGACACGGCGACT for *E. coli* RNAP and GGCGAGAGGGACACGGCGACT for T7 RNAP (lowercase indicates the halt position).

1. Lang K, Micura R (2008) The preparation of site-specifically modified riboswitch domains as an example for enzymatic ligation of chemically synthesized RNA fragments. *Nat Protoc* 3:1457–1466.
2. Roy R, Hohng S, Ha T (2008) A practical guide to single-molecule FRET. *Nat Methods* 5: 507–516.
3. Kapanidis AN, et al. (2004) Fluorescence-aided molecule sorting: Analysis of structure and interactions by alternating-laser excitation of single molecules. *Proc Natl Acad Sci USA* 101:8936–8941.
4. Lee S, Lee J, Hohng S (2010) Single-molecule three-color FRET with both negligible spectral overlap and long observation time. *PLoS One* 5:e12270.

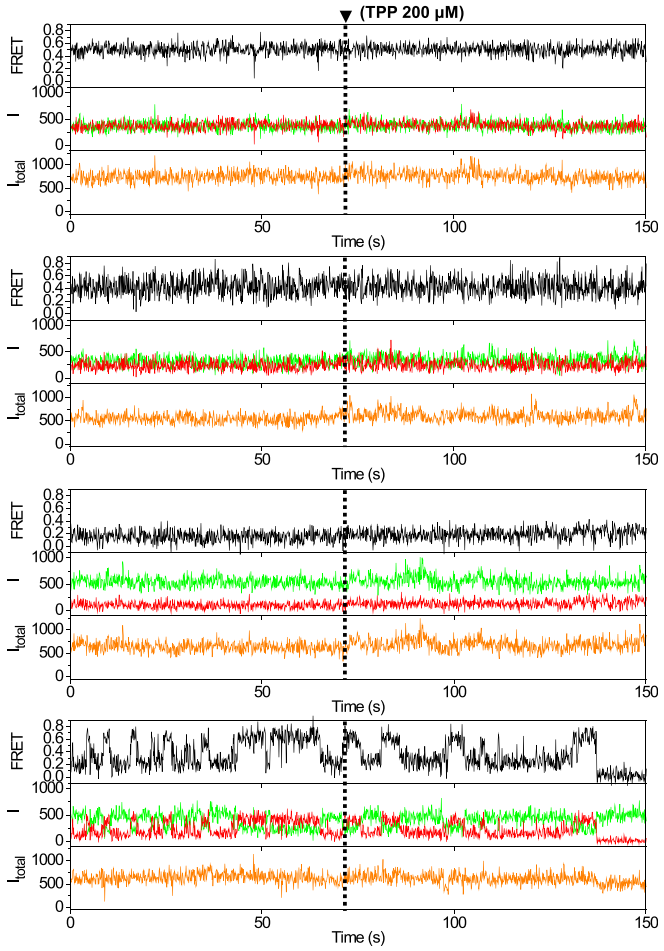

Fig. S1. Representative time traces of the thermally annealed synthetic aptamer that did not respond to TPP. Fluorescence intensities (I) of a donor and an acceptor are colored in green and red, respectively, and the corresponding FRET is shown in black. The total intensity (I_{total}) is shown in orange. TPP (200 μM) was injected at 70 s (dashed lines).

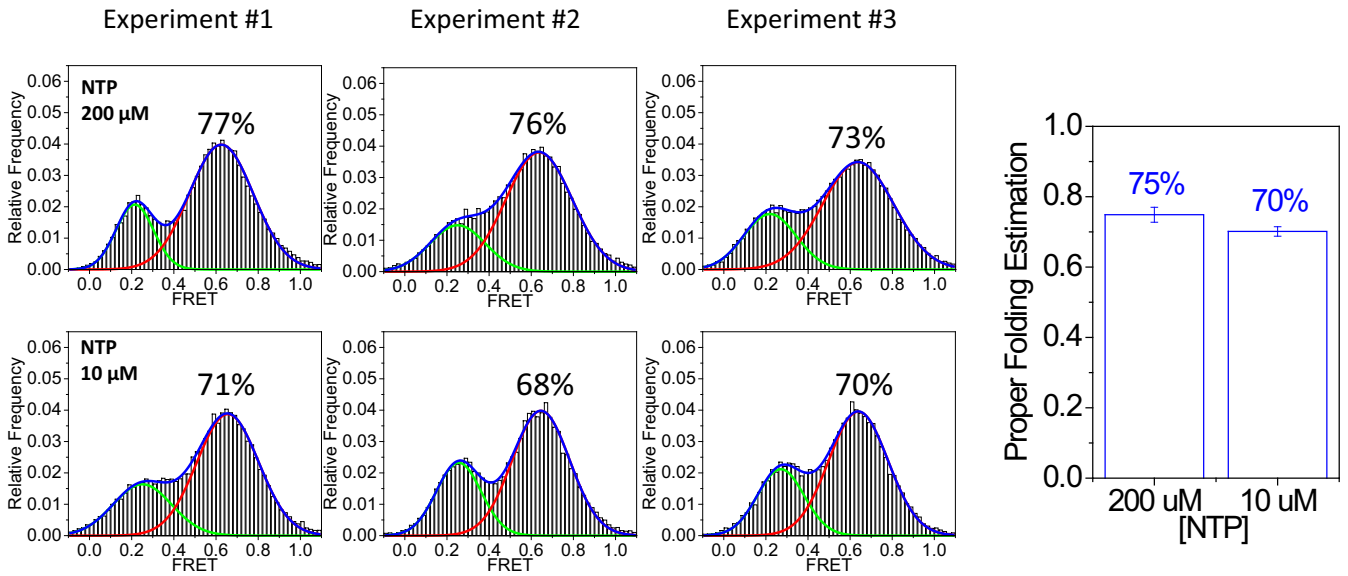

Fig. S2. Fraction of the high FRET population for D17 DNA at two different NTP concentrations for transcription (200 μM and 10 μM). The experiments were performed three times for each sample. (*Left*) FRET histograms were obtained after injection of 200 μM TPP. The total histograms are shown in Fig. 2*G*. (*Right*) As estimations of properly folded RNA, the fractions of the high FRET population were determined by fitting histograms to two Gaussian functions. Error bars represent the SD from three experiments, each with more than 50 molecules.

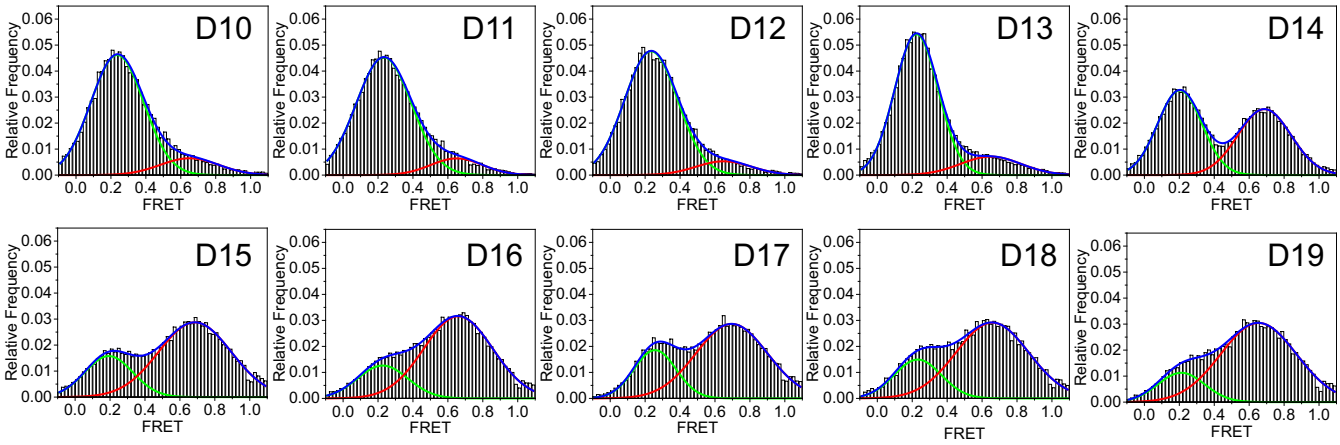

Fig. S3. Fraction of the high FRET population for D10–D19 DNA determined by fitting FRET histograms to two Gaussian functions. The experiments were performed three times for each sample, but only the total histograms are shown.

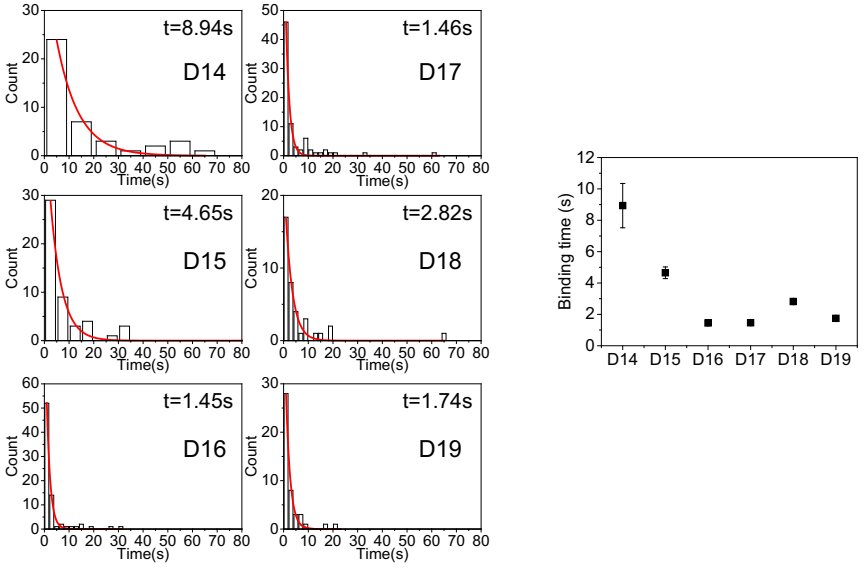

Fig. S4. TPP binding times for D14–D19 DNA. The binding times were measured after injecting 200 μM TPP. With D10–D13 DNA, binding events were too rare for data analysis to be performed.

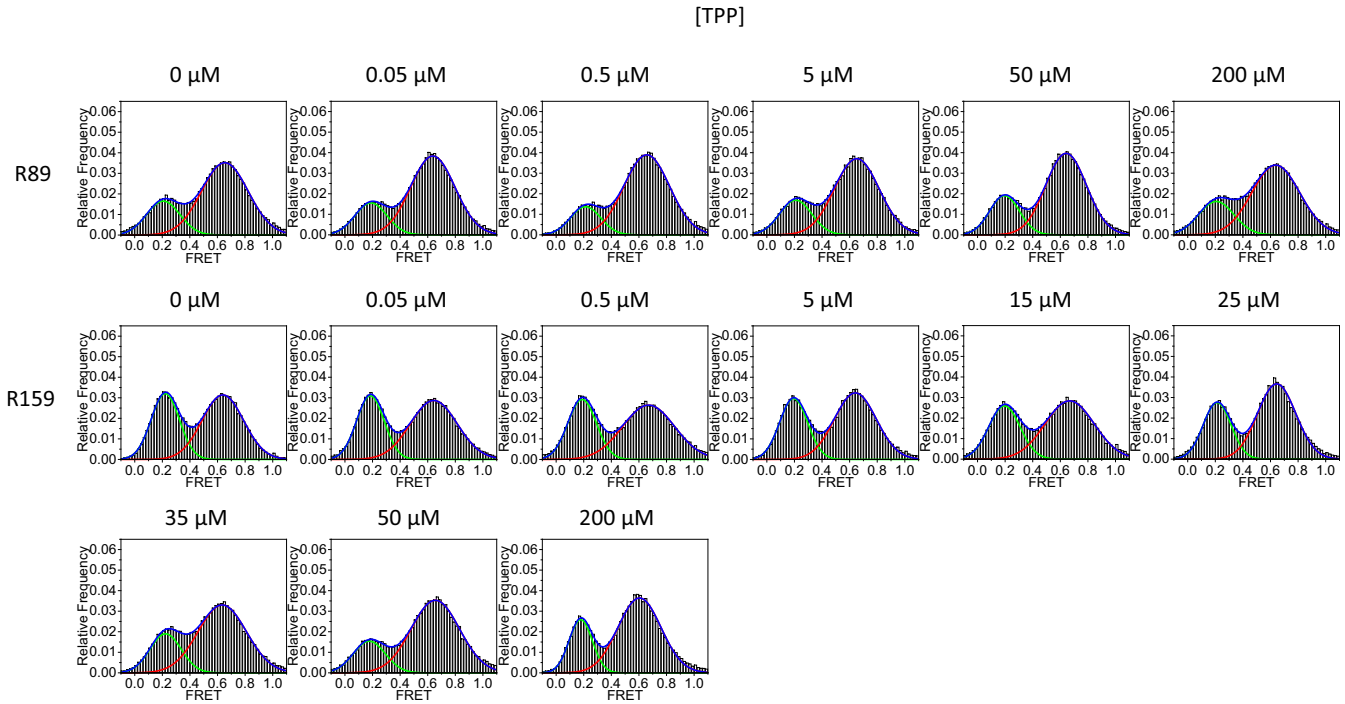

Fig. S5. FRET histograms for R89 and R159 RNAs with varying TPP concentrations during transcription. The FRET histograms were obtained after injecting 200 μM TPP, and were fitted to two Gaussian functions. The experiments were performed three times for each condition, but only the total histograms are shown.

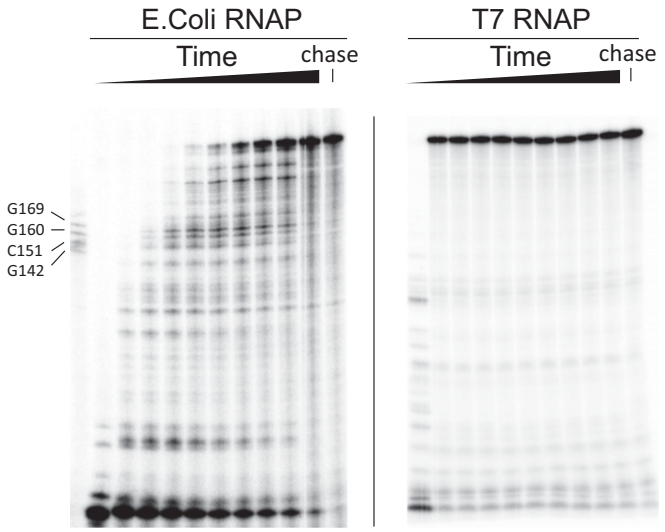

Fig. S6. Transcriptional pauses of *E. coli* and phage T7 RNAP. The ^{32}P-labeled ECs halted at position +20 were elongated through the TPP riboswitch. Samples were removed at 0, 15, 30, 45, 60, 75, 90, 105, 120, and 240 s and quenched. RNA products were separated on 6% denaturing polyacrylamide gel. The ladders on the left are transcripts elongated to the positions of G142, C151, G160, and G169, respectively.

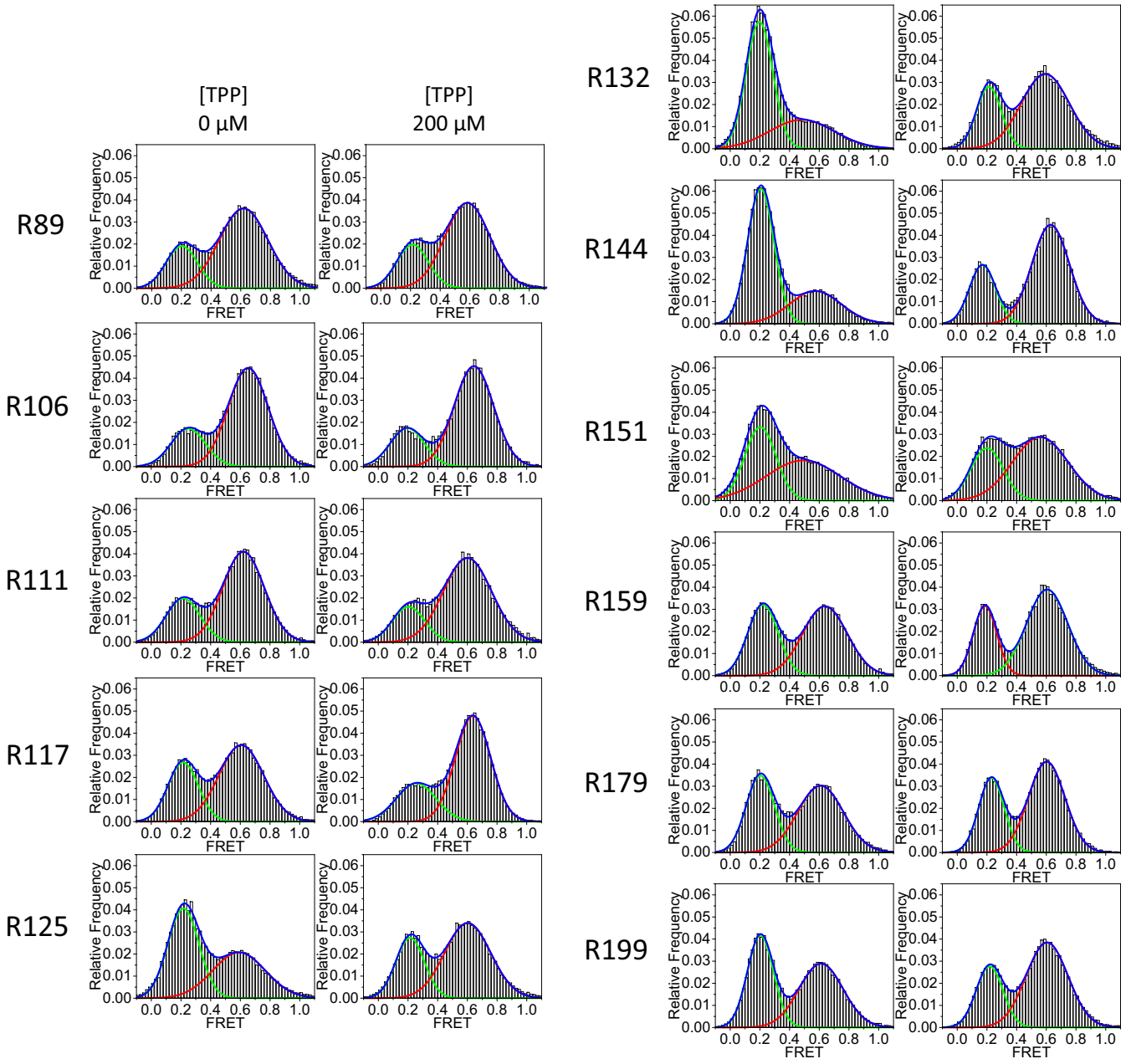

Fig. S7. FRET histograms for RNA transcripts of varying lengths. The RNA transcripts were synthesized either with or without 200 μM TPP. The FRET histograms were made in the presence of 200 μM TPP, and the fraction of the high FRET population was determined by fitting the histograms to two Gaussian functions. The experiments were performed three times for each sample, but only the total histograms are shown.

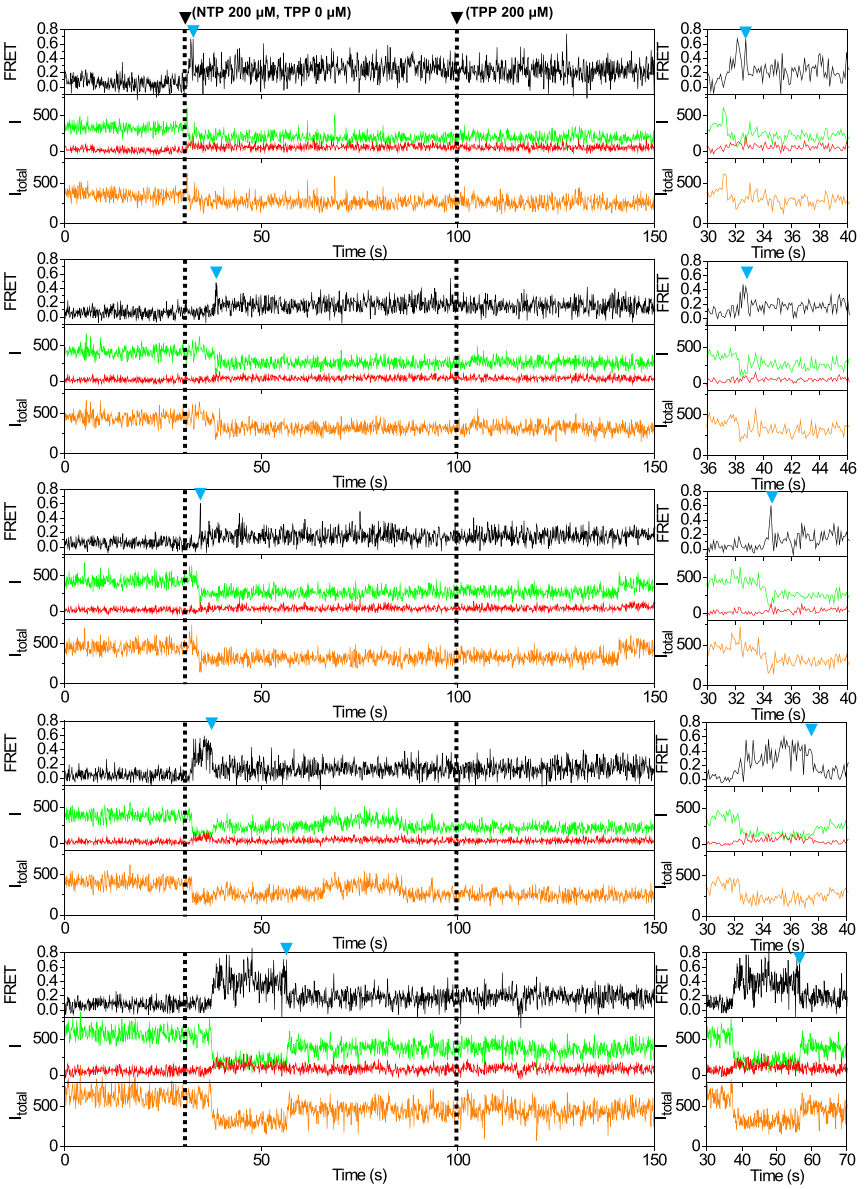

Fig. S8. Representative time traces that show off-to-on transitions (blue triangles) for R144 RNA. Fluorescence intensities (I) of a donor and an acceptor are colored in green and red, respectively, and the corresponding FRET is shown in black. The total intensity (I_{total}) is shown in orange. NTP (200 μM) and TPP (200 μM) were injected at 30 s and 100 s, respectively (dashed lines).

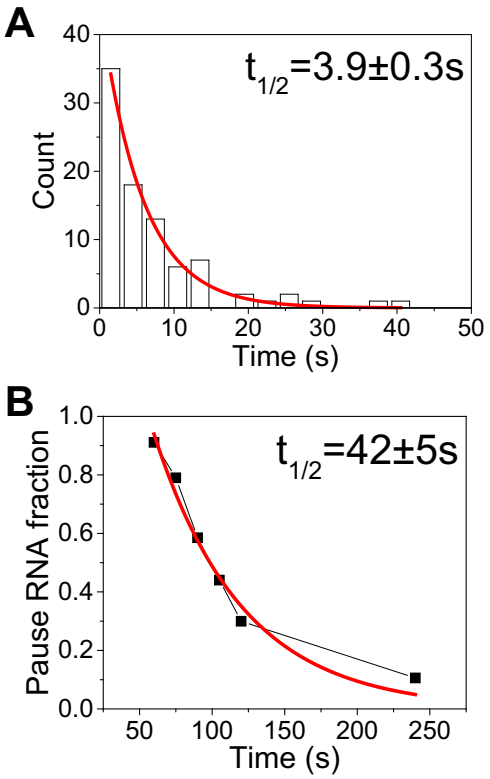

Fig. S9. Off-to-on transition time and transcriptional pausing time. (*A*) Transition time histogram from the off state to the on state for R144 RNA. (*B*) RNA fraction of the transcriptional pause for the time range from 60 to 240 s. Red lines are fits to single exponential functions. $t_{1/2}$, half-life.

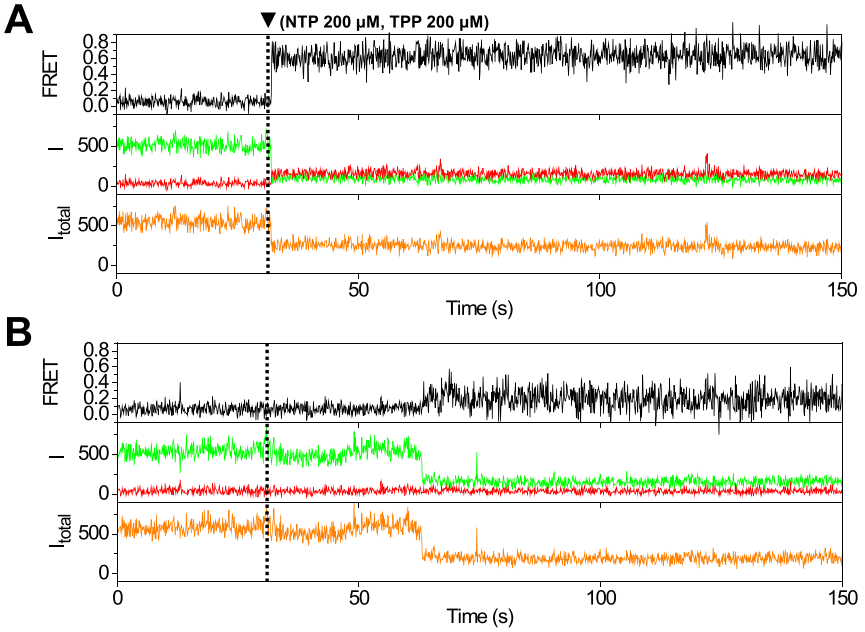

Fig. S10. Representative time traces of the off state (*A*) and the on state (*B*) for R144 RNA in the presence of 200 μM TPP. Fluorescence intensities (I) of a donor and an acceptor are colored in green and red, respectively, with the corresponding FRET shown in black. The total intensity (I_{total}) is shown in orange. NTP (200 μM) and TPP (200 μM) were injected at 30 s (dashed lines).

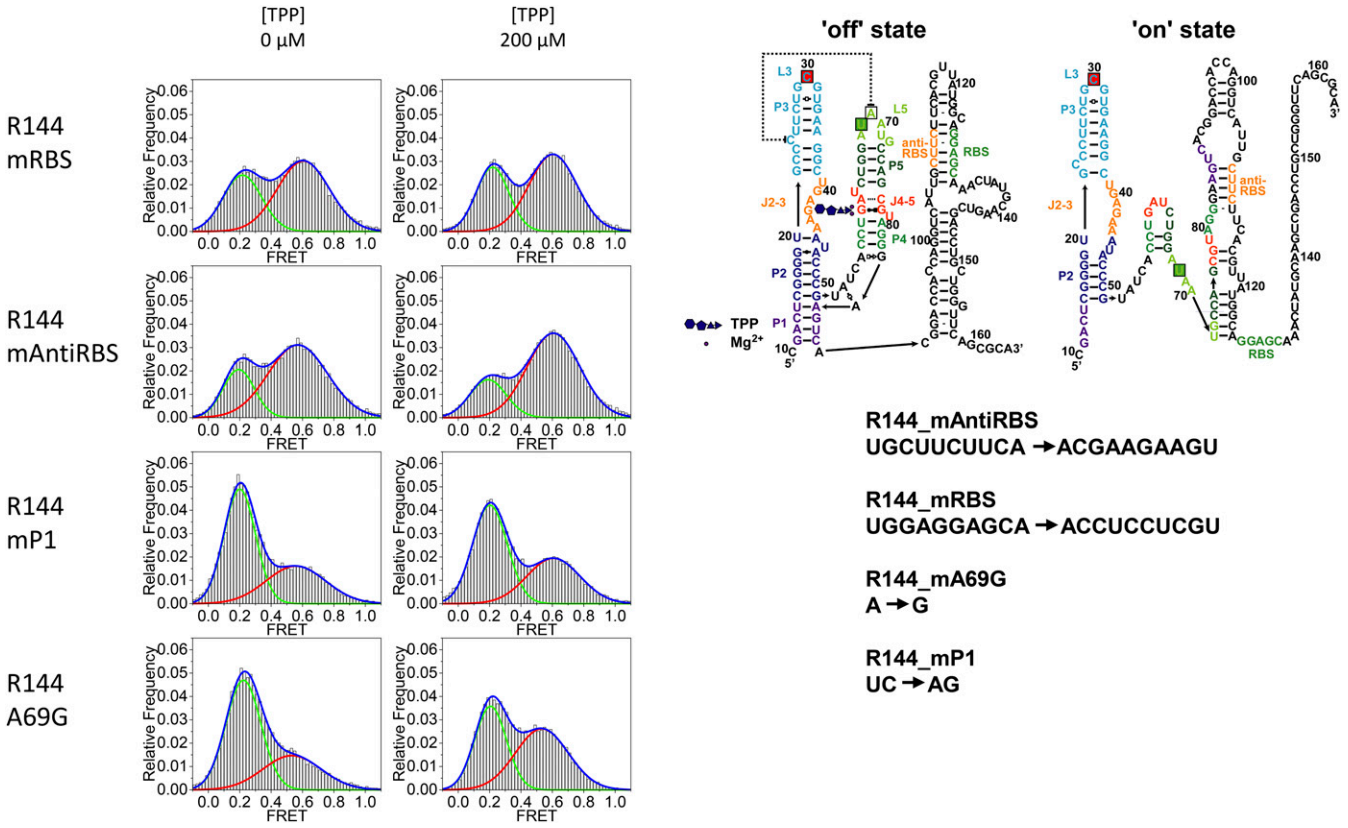

Fig. S11. Mutant samples and FRET histograms for different mutants. The RNA transcripts were synthesized either with or without 200 μM TPP. The FRET histograms were made in the presence of 200 μM TPP, and the fraction of the high FRET population was determined by fitting the histograms to two Gaussian functions. The experiments were performed three times for each sample, but only the total histograms are shown.

Table S1. Oligonucleotide sequences

Name	Sequence
Seed RNA	5′-ACGACUCGGGGUGCCCUUCUG-C(Dy647)-GUGAAGGCUGAGAAAUACCCGUAUCACCUGAUCUGGA-3′
Template DNA	5′-GCCACGAATGAGAAAGTCTAGACCTAAATGCCAGCGTAGGGAAGTCACGGACCACCAGGTCATTGCTTCTTCACGTTATGGCAGGAGCAAACTA-TGCAAGTCGACCTGCTGGGTTCAGCGCAATCTGCGCACGCGTTACACCTTTTTCACCAACATTCCCCCCGGAATTCCGG-3′
Nontemplate DNA	5′-Biotin-CCGGAATTCCGGGGGGAATGTTGGTGAAAAAGGTGTAACGCGTGCGCAGATTGCGCTGAACCCAGCAGGTCGACTTGCATAGTTTGCTC-CTGCCATAACGTGAAGAAGCAATGACCTGGTGGTCCGTGACTTCCCTACGCTGGCATTATCCAGATCACTTTCTCATTCGTGGC-3′

For the template and nontemplate DNAs, only the longest sequences are shown.