Supplementary Information for:

Pausing controls branching between productive and non-productive pathways during initial transcription

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Supplementary Protocols for the DNA constructs

The promoter DNA fragments (**Fig. S1A**) were prepared in a modular fashion, *i.e.* modular fragments of oligonucleotides (oligo 1, 2 and 3) were mixed with a complementary splint DNA to form a ligation-competent complex. Template and non-template strands were created separately. Oligo 2 was purchased from IBA Life Science (Göttingen, Germany), and the splint, oligo 1 and 3 were purchased from Sigma Aldrich (Poole, UK) and stored in TE buffer at -20°C (100µM).

DNA preparation: 100 pmol of each were mixed in 10 µl 1x T4 DNA ligase reaction buffer and phosphorylated at 37°C for 90 min using the T4 polynucleotide kinase (1,000 units/µl). The kinase was subsequently heat-inactivated. The oligonucleotides and the splint were mixed in 12 µl of 2x T7 DNA ligase buffer were added and annealed in a thermocycler. Finally, ligation was performed by T7 DNA ligase (120,000 units/µl) at 15°C over night. Enzymes and buffers were purchased from NEB.

Purification: to separate the ligated DNA strand from the splint and incomplete ligation products, we used a denaturing 10% polyacrylamide gel (14.5cm x 16.5cm x 1.5mm). While prerunning the gel in 1xTBE, the sample was mixed with formamide (85%), heated up to 95°C for 5 min, and immediately put on ice. The gel was run at 450V for 90min. Bands of the full-length product were excised and the strands eluted by crush-and-soak overnight and ethanol precipitated.

Template and non-template DNA strands assembly: the DNA promoters were created by annealing equimolar amounts of template and non-template strand oligonucleotides in buffer TE-50 (50 mM Tris pH 7.4, 1 mM EDTA, 50 mM NaCl) at 400 nM.

The oligonucleotides are represented in the 5' to 3' direction.

Oligonucleotide sequences for the non-template strands (name of the oligo, promoter, position within the promoter, length)

Splint WT and ds (-79,+25), 104 nt
GAAATTGTTATCCGCTCTCACAATTCCACACATTATACGAGCCGAAGCATAAAGTGTCAAGCCTGGGGTGC
CTAAAATGTGATCTAGATCACATTTATTGCGTT

Splint -7T/A (-79,+25), 104 nt
GAAATTGTTATCCGCTCTCACAATTCCACACTTTATACGAGCCGAAGCATAAAGTGTCAAGCCTGGGGTGC
CTAAAATGTGATCTAGATCACATTTATTGCGTT

Splint WT ITC6 (-79,+25), 104 nt
GAAATTGTTATCCGCTCCTACAATTCCACACATTATACGAGCCGAAGCATAAAGTGTCAAGCCTGGGGTGC
CTAAAATGTGATCTAGATCACATTTATTGCGTT

Splint ΔP (-79,+25), 104 nt
GAAATTGTTATCCGCTCTACCAATTCCACACATTATACGAGCCGAAGCATAAAGTGTCAAGCCTGGGGTGC
CTAAAATGTGATCTAGATCACATTTATTGCGTT

Oligo 3 (-89,-30), 60 nt
CAGTGAGCGCAACGCAATAAATGTGATCTAGATCACATTTTAGGCACCCCAGGCTTGACA

Oligo 2 (-29,-10), 20 nt
CTTTATGCTTCGGCXCGTAT with X=T-Cy3B

Oligo 1 WT and ds (-9,+25), 34 nt
AATGTGTGGAATTGTGAGAGCGGATAACAATTTC

Oligo 1 -7T/A (-9,+25), 34 nt
AAAGTGTGGAATTGTGAGAGCGGATAACAATTTC

Oligo 1 WT ITC6 (-9,+25), 34 nt
AATGTGTGGAATTGTAGGAGCGGATAACAATTTC

Oligo 1 ΔP (-9,+25), 34 nt
AATGTGTGGAATTGGTAGAGCGGATAACAATTTC

Oligonucleotides for WT template strand

Splint (-79,+25), 104 nt
AACGCAATAAATGTGATCTAGATCACATTTTAGGCACCCCAGGCTTGACACTTTATGCTTCGGCTCGTAGC
CGTGTTGGAATTGTGAGAGCGGATAACAATTTC

Oligo 1 (+8,+25), 18 nt
GAAATTGTTAXCCGCTCT with X=T-ATTO647N

Oligo 2 (-29,+7), 36 nt
CACAATTCCAACACGGCTACGAGCCGAAGCATAAAG

Oligo 3 (-89,-30), 60 nt
TGTCAAGCCTGGGGTGCCTAAAATGTGATCTAGATCACATTTATTGCGTTGCGCTCACTG

Oligonucleotides for WT ITC6 template strand

Splint (-79,+25), 104 nt
AACGCAATAAATGTGATCTAGATCACATTTTAGGCACCCCAGGCTTGACACTTTATGCTTCGGCTCGTAGC
CGTGTTGGAATTGTAGGAGCGGATAACAATTTC

Oligo 1(+8,+25), 18 nt
GAAATTGTTAXCCGCTCC with X=T-ATTO647N

Oligo 2 (-29,+7), 36 nt
TACAATTCCAACACGGCTACGAGCCGAAGCATAAAG

Oligo 3 (-89,-30), 60 nt
TGTCAAGCCTGGGGTGCCTAAAATGTGATCTAGATCACATTTATTGCGTTGCGCTCACTG

Oligonucleotides for ΔP *template strand*

Splint (-79,+25), 104 nt
AACGCAATAAATGTGATCTAGATCACATTTTAGGCACCCCAGGCTTGACACTTTATGCTTCGGCTCGTAGC
CGTGTTGGAATTGGTAGAGCGGATAACAATTTC

Oligo 1 (+8,+25), 18 nt
GAAATTGTTAXCCGCTCT with X=T-ATTO647N

Oligo 2 (-29,+7), 36 nt
ACCAATTCCAACACGGCTACGAGCCGAAGCATAAAG

Oligo 3 (-89,-30), 60 nt
TGTCAAGCCTGGGGTGCCTAAAATGTGATCTAGATCACATTTATTGCGTTGCGCTCACTG

Oligonucleotides for ds template strand

Splint (-79,+25), 104 nt
AACGCAATAAATGTGATCTAGATCACATTTTAGGCACCCCAGGCTTGACACTTTATGCTTCGGCTCGTATA
ATGTGTGGAATTGTGAGAGCGGATAACAATTTC

Oligo 1 (+8,+25), 18 nt

GAAATTGTTAXCCGCTCT with X=T-ATTO647N

Oligo 2 (-29,+7), 36 nt
CACAATTCCACACATTATACGAGCCGAAGCATAAAG

Oligo 3 (-89,-30), 60 nt
TGTCAAGCCTGGGGTGCCTAAAATGTGATCTAGATCACATTTATTGCGTTGCGCTCACTG

Supplementary Protocols for in vitro bulk transcription experiments

Enzymes & transcription templates. For *in vitro* transcription reactions, RNAP holoenzyme (Epicentre or New England Biolabs) was purchased and used without further purification. Sigma 70 was purified as described (Kulbachinskiy and Mustaev, 2006) and incubated at 2-fold excess with the core enzyme for 30 min at 33 ºC to form a holoenzyme, which was stored in RNAP storage buffer (20 mM Tris-HCl pH 7.9, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 50% v/v Glycerol) at -20 ºC until needed. Sequences of transcription promoter-templates and oligos are provided in **Figure S1A**. The transcription template used here was a shorter version of the WT promoter (**Fig. S1A**), from -39 to +25, and was created by annealing equimolar amounts of template and non-template strand oligonucleotides in buffer TE-50 (50 mM Tris pH 7.4, 1 mM EDTA, 50 mM NaCl) at 100-400 nM as required.

***In vitro* transcription reactions.** For control experiments on bead-bound transcription complexes, 2.5 µl streptavidin-coated magnetic beads (DynaBeads, Life Technologies) were equilibrated in 1x S-2 KG7 buffer, pre-formed RP_O was bound at 37ºC for 15 min with gentle agitation, and beads were washed 3x with 1x KG7. Transcription was initiated by addition of 4 µl reaction mix and agitated gently. 1x reaction mix contains: 4 units of RNAsin (Promega, USA), 0.1 mg/mL heparin, 500 µM dinucleotide primer (e.g. ApA) and either 80 µM each NTP for a subset to reach 7 nt (e.g. UTP + GTP for lac) or all four NTPs (runoff transcription) in 1x KG7 buffer. The reaction mix was then supplemented with 0.6 µCi/µL [α-^{32}P]-UTP (Perkin Elmer). For measurements of total transcript, FLB-stop buffer (86% formamide, 20 mM EDTA, 1x TBE, amaranth dye) was added to the beads directly to quench the reactions and the mixture was heated to 95ºC for 4 min, placed on ice, and the supernatant was loaded directly onto a high-resolution sequencing gel (30% 19:1 acrylamide/bis-acrylamide gel, 6 M urea, 1x TBE) and electrophoresed in 1x TBE at 32 V/cm for 30 min until samples entered the gel, then at 75 V/cm until the amaranth dye had run ~85% of the gel length. Typical running time for a 40-cm gel was 3000 V for 3.5 hours. The gel was cut to remove unincorporated radioactive NTPs (typically 1 cm above the amaranth dye band).

For released vs. retained experiments, reactions were halted after a 10-sec incubation by pulling down the transcription complexes, then one time rinsed with KG7 1x buffer and disposed in KG7 1x buffer for the indicated duration. Subsequently, the transcription complexes were pulled-down, to run on a PAGE gel the released transcripts collected from the supernatant (Released Fraction, **Fig. S4**) and the transcripts retained in the transcription complexes (Retained Fraction, **Fig. S4**).

Table S1: Statistics, exit rates and probabilities for all the experiments performed in single-molecule FRET.

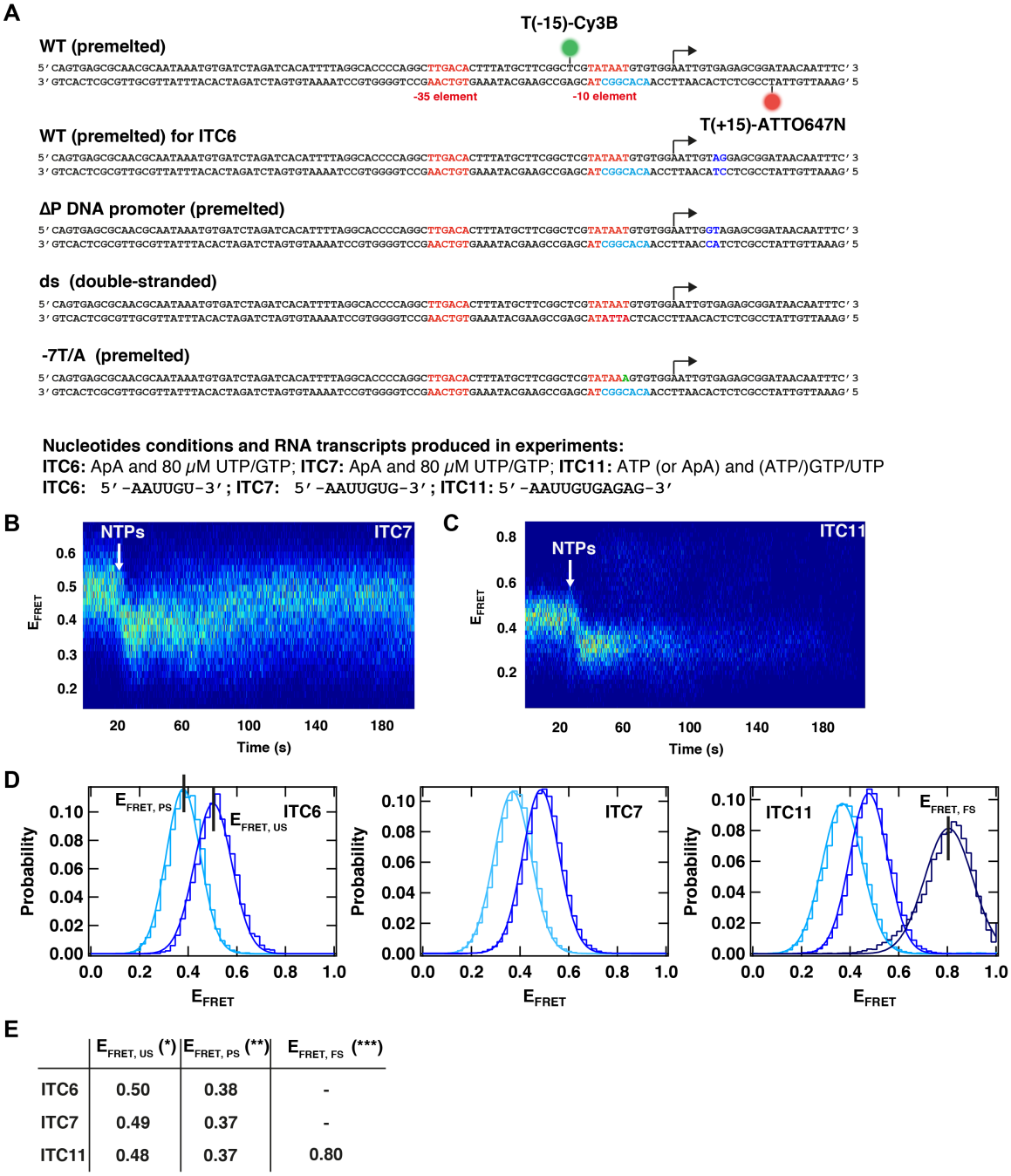
Experimental conditions (promoter, core RNAP, σ, starting substrate, NTPs, rinsed)	Figures	Number of traces; with FS FRET levels	IPE number of points; k_{IPE} (s^{-1})	US number of points; k_1 (s^{-1}); k_2 (s^{-1}); $P(k_1)$	PS number of points; k_1 (s^{-1}); k_2 (s^{-1}); $P(k_1)$	FS number of points; k_1 (s^{-1}); k_2 (s^{-1}); $P(k_1)$
WT, WT, WT, ApA, all NTPs 1µM, no	2, 4, S2	151; 8	8; (*)	311; 0.11; 0.013;0.5	382; 0.11; 0.012;0.64	(**)
WT, WT, WT, ApA, all NTPs 10µM, no	2, 4, S2	176; 44	38; 0.046	223; 0.09; 0.014;0.57	278; 0.27; 0.013;0.21	(**)
WT, WT, WT, ApA, all NTPs 30µM, no	2, 4, S2	317; 135	123; 0.041	238; 0.17; 0.017;0.65	283; 0.13; 0.015;0.25	(**)
WT, WT, WT, ApA, all NTPs 50µM, no	2, 4, S2	310; 162	148; 0.037	184; 0.20; 0.032;0.63	218; 0.24; 0.017;0.20	(**)
WT, WT, WT, ApA, all NTPs 80µM, no	2, 4, S2	599; 363	308; 0.049	313; 0.22; 0.029;0.46	262; 0.22; 0.019;0.07	(**)
WT, WT, WT, ApA, ITC11 80µM, no	3	238; 167	149; 0.052	(**)	(**)	(**)
WT, WT, WT, ApA, all NTPs 500µM, no	2, 4, S2	330; 266	233; 0.046	(*)	(*)	(**)
WT, WT, WT, ATP, ITC11 5µM, no	2, 4, S2	94; 28	18; 0.079	151; 0.14; 0.018;0.49	176; 0.10; 0.011;0.31	(**)
WT, WT, WT, ATP, ITC11 10µM, no	2, 4, S2	88; 33	29; 0.094	103; 0.16; 0.014;0.46	133; 0.12; 0.011;0.57	(**)
WT, WT, WT, ATP, ITC11 30µM, no	2, 4, S2	230; 143	121; 0.10	151; 0.08; 0.012;0.68	174; 0.10; 0.018;0.47	(**)
WT, WT, WT, ATP, ITC11 50µM, no	2, 4, S2	211; 122	94; 0.115	160; 0.10; 0.014;0.55	177; 0.07; 0.014;0.58	(**)
WT, WT, WT, ATP, ITC11 80µM, no	2, 4, S2	246; 150	128; 0.099	139; 0.12; 0.014;0.56	159; 0.08; 0.015;0.65	(**)
WT, WT, WT, ATP, ITC11 80µM, no	*5, S3*	*79; (**)*	*(**)*	*681; 0.93; 0.057;0.87*	*115; 0.59; 0.045;0.36*	*596; 0.32; 0.059; 0.81*
WT, WT, WT, ATP, ITC11 500µM, no	2, 4, S2	191; 141	120; 0.155	(*)	(*)	(**)
ΔP, WT, WT, ATP, ITC11 5µM, no	2, 4, S2	88; 55	42; 0.18	(*)	(*)	(**)
ΔP, WT, WT, ATP, ITC11 10µM, no	2, 4, S2	94; 60	53; 0.19	(*)	(*)	(**)
ΔP, WT, WT, ATP, ITC11 30µM, no	2, 4, S2	85; 66	52; 0.29	(*)	(*)	(**)
ΔP, WT, WT, ATP, ITC11 50µM, no	2, 4, S2	245; 190	147; 0.21	(*)	(*)	(**)
ΔP, WT, WT, ATP, ITC11 80µM, no	2, 4, S2	152; 123	94; 0.32	(*)	(*)	(**)
ΔP, WT, WT, ATP, ITC11 500µM, no	2, 4, S2	97; 76	60; 0.33	(*)	(*)	(**)
ΔP, WT, WT, ApA, all NTPs 1µM, no	2, 4, S2	96; 3	3; (*)	238; 0.15; 0.018;0.53	289;0.096; 0.018;0.53	(**)
ΔP, WT, WT, ApA, all NTPs 5µM, no	2, 4, S2	148; 49	39; 0.057	285; 0.22; 0.020;0.47	327;0.28; 0.024;0.27	(**)
ΔP, WT, WT, ApA, all NTPs 10µM, no	2, 4, S2	162; 71	66; 0.064	189; 0.12; 0.013;0.76	216; 0.098; 0.020;0.46	(**)
ΔP, WT, WT, ApA, all NTPs 30µM, no	2, 4, S2	328; 220	186; 0.051	210; 0.14; 0.026;0.59	249; 0.20; 0.030;0.36	(**)
ΔP, WT, WT, ApA, all NTPs 50µM, no	2, 4, S2	311; 210	172; 0.06	213; 0.19; 0.025;0.68	237; 0.11; 0.025;0.56	(**)
ΔP, WT, WT, ApA, all NTPs 80µM, no	2, 4, S2	302; 209	164; 0.085	123; 0.13; 0.015;0.7	130; 0.10; 0.025;0.62	(**)
ΔP, WT, WT, ApA, all NTPs 500µM, no	2, 4, S2	326; 258	226; 0.075	87;0.19;0.04;0.89	109;0.08;0.026;0.56	(**)
WT, WT, F522A, ApA, ITC11 80µM, no	3, S2	207; 78	67; 0.047	107; 0.12; 0.018;0.75	134; 0.10; 0.022;0.45	(**)
WT, WT, F522A, ATP, ITC11 80µM, no	S2	289; 119	49; 0.15	133; 0.21; 0.024;0.63	153; 0.34; 0.024;0.67	(**)
WT, D446A, WT, ApA, ITC11 30µM, no	S2	53; 6	6; (*)	(*)	(*)	(**)
WT, D446A, WT, ATP, ITC11 30µM, no	S2	37; 12	12; (*)	121; 0.15; 0.031;0.51	145; 0.19; 0.017;0.70	(**)
WT, D446A, WT, ApA, ITC11 500µM, no	3, S2	127; 27	15; 0.014	121; 0.15; 0.031;0.51	145; 0.19; 0.017;0.70	(**)
WT, D446A, WT, ATP, ITC11 500µM, no	S2	137; 47	29; 0.055	158; 0.13; 0.018;0.67	179; 0.13; 0.016;0.65	(**)
-7T/A, WT, WT, ApA, ITC11 80µM, no	S2	256; 139	131; 0.036	169; 0.13; 0.016;0.82	196; 0.29; 0.018;0.56	(**)
dsWT, WT, WT, ApA, ITC11 80µM, no	S2	148; 21	21; 0.037	313; 0.22; 0.029;0.46	262; 0.22; 0.019;0.07	(**)
dsWT, WT, WT, ATP, ITC11 80µM, no	S2	132; 77	64; 0.104	(*)	(*)	(**)
WT, WT, WT, ApA, ITC7 80µM, no	S2	221; 0	(***)	544; 0.19; 0.024;0.53	633; 0.22; 0.022;0.15	(**)
WT, WT, WT, ApA, ITC7 80µM, yes	5, S3	70; 0	(***)	225; 0.11; 0.023;0.60	209; 0.14; 0.019;0.51	(**)
WT, WT, WT, ApA, ITC11 80µM, yes	5, S3	99; 48	(***)	244; 0.10; 0.016;0.65	208; 0.15; 0.019;0.42	(***)
WT, WT, WT, ApA, ITC11 80µM, yes	5, S3	99; 48	(***)	396; 1.0; 0.06;0.78	84; 0.12; 0.028;0.59	313; 0.36; 0.075;0.64
WT, WT, WT, ATP, ITC11 80µM, yes	5, S3	129; 79	(***)	383; 0.26; 0.031;0.52	402; 0.26; 0.024;0.78	(***)
WT, WT, WT, ATP, ITC11 80µM, yes	5, S3	129; 79	(***)	763; 0.95; 0.081;0.73	236; 0.39; 0.049;0.67	617; 0.68; 0.091;0.71

(*) Not determined because the statistics was insufficient

(**) Not Relevant.

(***) Not existing in these experimental conditions.

In italic: same dataset as the line above, kinetics after reaching the FS FRET level the first time.



E

	$E_{FRET, US}$ (*)	$E_{FRET, PS}$ (**)	$E_{FRET, FS}$ (***)
ITC6	0.50	0.38	-
ITC7	0.49	0.37	-
ITC11	0.48	0.37	0.80

(*) US: unscrunched; (**) PS: partly scrunched; (***) FS: fully scrunched.

Fig. S1. DNA promoter sequences and initial transcribing single-molecule FRET calibration. (A) The different DNA promoter sequences used in the study. The arrow indicate the start site and the light blue font indicates the premelted region. **(B)** Transcription heatmap in ITC7 condition (500 μM ApA and 80 μM GTP-UTP) using the WT DNA construct. **(C)** Transcription heatmap in ITC11 condition (500 μM ApA and 80 μM GTP/UTP/ATP) using the WT DNA construct. **(D)** Histograms reporting the FRET efficiency E_{FRET} for each FRET level, i.e. low, intermediate and high represented in light blue, blue and dark blue respectively, in each ITC conditions, i.e. ITC6, ITC7 and ITC11. The solid lines are Gaussian fits that provides the center of each distribution. **(E)** FRET levels center extracted from the Gaussian fit performed in (D) for the FRET levels of the US, PS and FS states for each Initial Transcribing Complex (ITC).

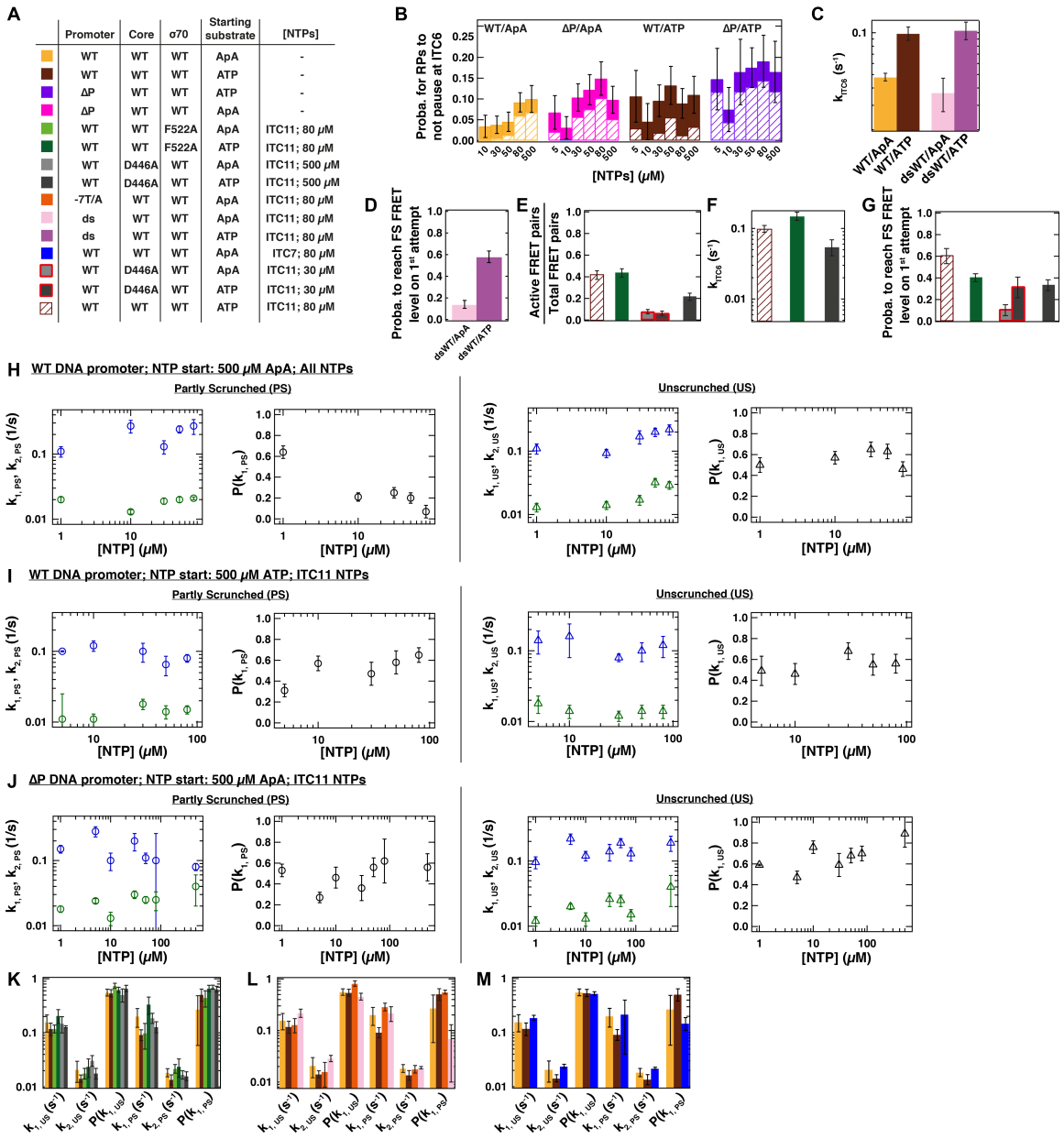
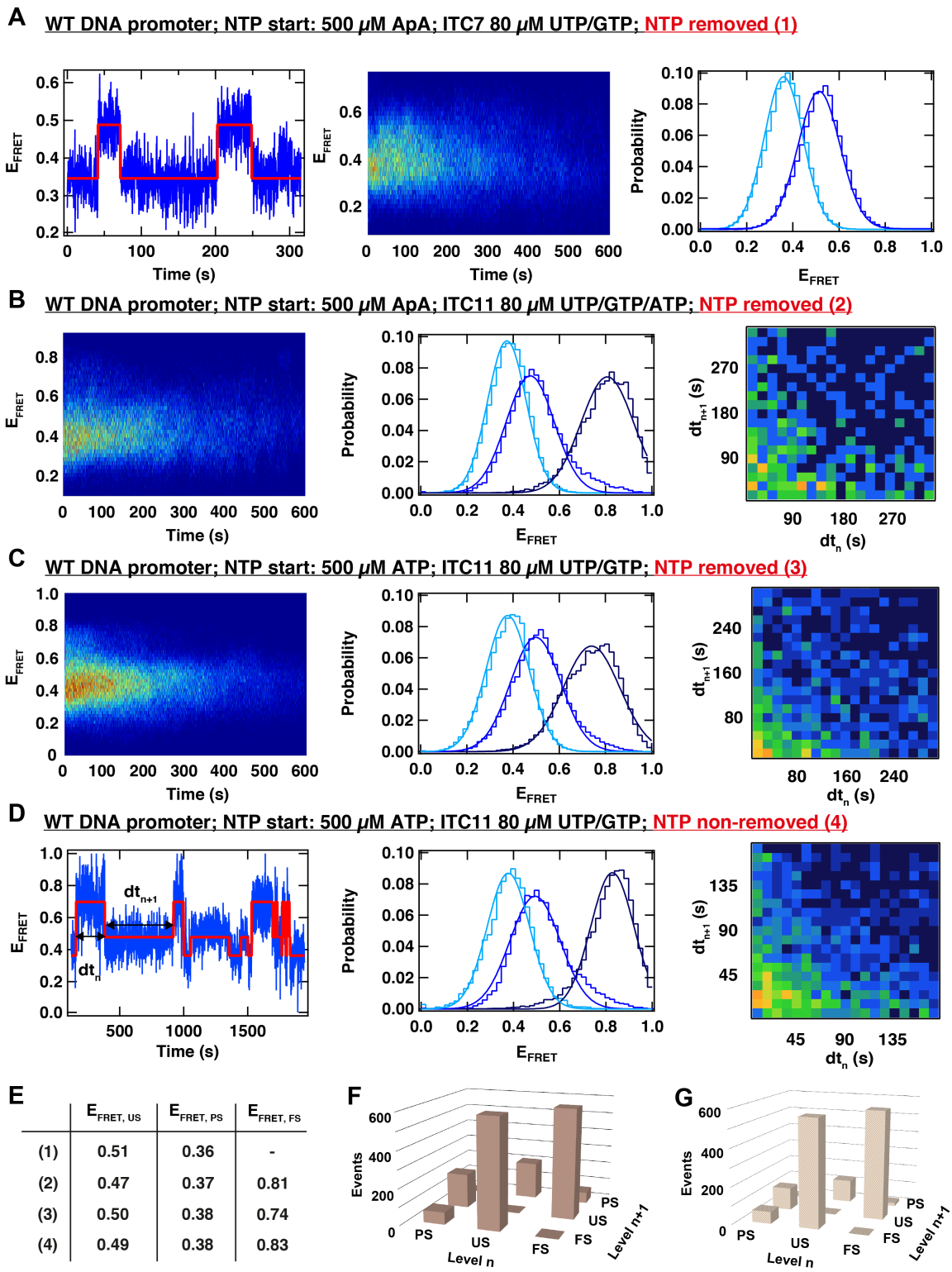


Fig. S2. Kinetics of the ITC6 pause and the US and PS FRET states for different NTPs concentrations, different initiating nucleotides, dsWT promoter, and core and σ^{70} mutants. Starting substrate is either ApA or ATP, at 500 μM concentration. **(A)** Table with the different experimental variables explored in (B)-(J) and the color code associated. Data represented in yellow and brown in (H)-(J) are averaged values for all NTP concentrations (see Fig. 2A), and in (C) the individual values for each concentration of NTPs (B). **(B)** Probability to reach the FS FRET level without pausing at ITC6. The dashed bars are the data corrected from the fraction of the ITC6 pause exponential distribution that is faster than our time limit (the frame time, i.e. 80 ms for ΔP and ATP and 200 ms otherwise, times two as we keep pauses longer or equal to two frames). The data are extracted from **Table S1**. The error bars are values at 95% confidence interval. **(C)** Exit rate k_{ITC6} (**Fig. 1D**) using the WT DNA promoter sequence or the dsWT promoter (**Fig. S1A**) as a function of NTP start. Data from **Fig. 2D** (yellow and brown) are represented here for comparison. **(D)** Probability to escape the ITC6 pause on the first attempt as in **Fig. 1D** for the conditions in (C). **(E)** Ratio of the number of FRET pairs, i.e. a pair of made of a single acceptor dye and a single donor dye that demonstrate FRET (see **Materials and Methods: FRET pair localization and detection**), showing transcription activity over the total number of FRET pairs detected for the experimental conditions described in (A). **(F)** ITC6 pause exit rate for the

experimental conditions described in (A). **(G)** Probability to reach the FS FRET level on the 1st attempt for the experimental conditions described in (A). **(H)-(J)** Parameters from a two-exponential Maximum Likelihood Estimation (MLE) fit of the distribution of the dwell times from the US and PS FRET levels from the complexes that did not reach the FS FRET level on the first attempt (**Fig. 1D**) as a function of the concentration of NTPs: exit rates $k_{1, PS}$ (blue circles), $k_{2, PS}$ (green circles) and $P(k_{1, PS})$ the probability to exit with $k_{1, PS}$ for the PS FRET level; exit rates $k_{1, US}$ (blue triangles), $k_{2, US}$ (green triangles) and $P(k_{1, US})$ the probability to exit with $k_{1, US}$ for the US FRET level. **(K, L, M)** Parameters extracted from a double-exponential MLE fitting of the US and PS FRET levels as described in (H)-(J) for the experimental conditions described in (A).



	$E_{FRET, US}$	$E_{FRET, PS}$	$E_{FRET, FS}$
(1)	0.51	0.36	-
(2)	0.47	0.37	0.81
(3)	0.50	0.38	0.74
(4)	0.49	0.38	0.83

Fig. S3: Nascent RNA is retained within the transcription complex when the scrunched downstream DNA promoter is relaxed. The experiments here are performed with the WT DNA promoter sequence (**Fig. S1A**). **(A)** FRET trace of transcription complex that has been incubated in ITC7 conditions followed by NTP removal as described in **Fig. 4A**, transcription heatmap and histogram of the PS (light blue) and US (blue) FRET levels. **(B)** and **(C)** transcription complexes alternate between US, PS and FS FRET levels after NTP removal after incubation in ITC11 condition (**Fig. 4A**) with either ApA (B) or ATP (C) as starting substrate. Here is represented a transcription heatmap, the FRET levels histograms and their respective Gaussian fits (solid line)

and a correlation map. The correlation map represents the duration of the dwell time dt_n as a function of the duration of the subsequent dwell time dt_{n+1} (time trace in (D)). **(D)** As in (B) and (C) but in the presence of NTPs (ITC11 condition, ATP starting substrate and 80 µM NTPs) and after reaching the FS FRET level on the first attempt. The FRET levels histograms and their respective Gaussian fits (solid line) are represented in the second panel and the correlation map of two by two successive dwell times dt, as in (B) and (C). **(E)** FRET levels average values extracted from the peak position of the Gaussian fits performed in (A), (B), (C) and (D) for the US, PS and FS FRET levels. **(F), (G)** 3D histogram showing the number of transitions occurring between the different FRET levels for the n and n+1 successive dt, for the experiments described in (C) and (D).

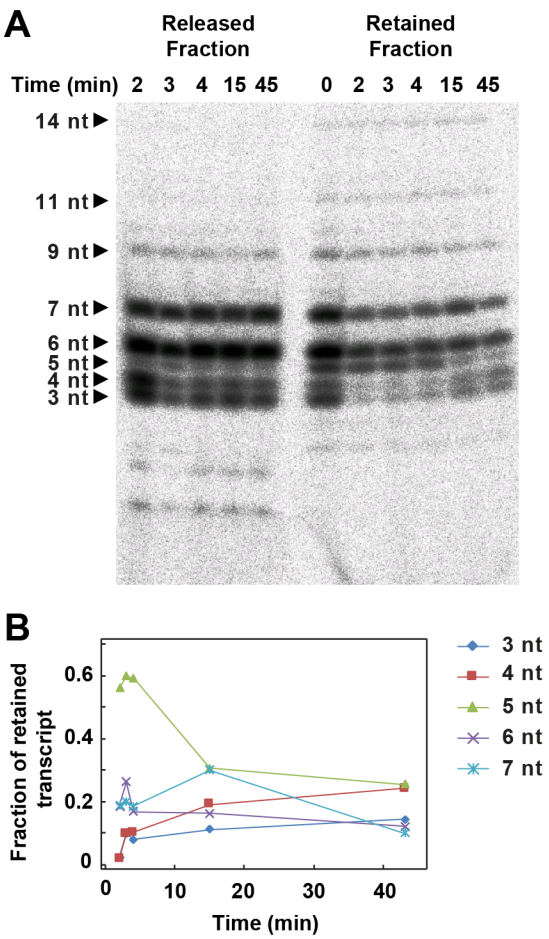


Fig. S4: Bead-attached ITCs forms a stable complex with the nascent RNA that is competent for elongation. (A) Gel electrophorisis assay showing the part of the nascent RNA trancripts that is released and retained as a function of the waiting time after NTP removal (ITC7 conditions, see **Supplementary Text**). **(B)** Analysis of the gel from (A) showing the fraction of the retained transcript as a function of time and length of the transcript. The apparent increase in the fraction of retained 3- and 4-nt RNA between 15 and 45 min is explained by the cleavage of the 5 nt RNA by the nuclease activity of the RNAP.

References

Kulbachinskiy, A., and Mustaev, A. (2006). Region 3.2 of the sigma subunit contributes to the binding of the 3'-initiating nucleotide in the RNA polymerase active center and facilitates promoter clearance during initiation. The Journal of biological chemistry *281*, 18273-18276.