Supporting Information

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SI Text

SI Materials and Methods. ***Enzyme and templates.*** His_6-tagged Δ-loop RNA polymerase (RNAP) was made by site-directed mutagenesis of the pRL-706 plasmid (gift of R. Landick). Expression was carried out in the *Escherichia coli* strain TOM100 (gift of T. Santangelo) and purified as previously described (1).

Transcription elongation experiments. To form open promoter complexes (OPCs), RNAP (80 nM) was combined with template DNA (80 nM) bound to streptavidin magnetic beads in 1× transcription buffer [30 mM Hepes-NaOH (pH 8.0), 10 mM $Mg^{<+2}$ glutamate, 200 mM K^+ glutamate, 25 μg/mL BSA, and 1 mM DTT] and incubated at 37 °C for 10 min. After incubation, stalled elongation complexes (SECs) at position +24 were formed by the addition of a mixture of 20 μM ATP, 15 μM UTP, 16 μM GTP, and 4 μM [α-^{32}P]-GTP (800 Ci/mmol) to the OPCs and incubating for 2 min at room temperature. SECs were purified from free nucleoside triphosphates (NTPs) and resuspended in 1× transcription buffer prior to loading into a Kintek Rapid Quench 3 device (Kintek Corporation, Austin, TX).

Rapid quench experiments. The purified complexes were kept on ice until used in the experiments. Reactions were mixed (20 μL of complexes and 20 μL NTPs) in the Rapid Quench for the desired time prior to quenching with 0.5 M EDTA or 1 M HCl. To make sure that the time between time points while conducting the experiments did not affect the results, the order in which the time points were collected was randomized. In addition, to ensure that complexes were active throughout the experiments, a small aliquot of purified SECs was extended to full length by the addition of all 1 mM of all 4 NTPs. Samples were electrophoresed on an 8 M urea, 20% polyacrylamide gel and quantified using ImageQuant v5.2. Excess quench and buffer were removed from the EDTA quenched samples by retaining the complexes with a magnet, and then the complexes were denatured with formamide before loading on the gel. The HCl quenched samples were precipitated with ethanol and then resuspended in formamide before loading on the gel. Experiments involving a different template identity were performed as described above with the exception that the SECs were "walked" to position +26 by the addition of 10 μM CTP and 10 μM ATP. Complexes were allowed to react for 1 min and the free NTPs were removed and the complexes washed with ice-cold 1× transcription buffer. The resulting cleaned SECs stalled at +26 were then used as described for the preincubation and simultaneous addition experiments with GTP and CTP.

Shuttle experiments. The NTP loading experiments were performed as follows. Unlabeled SECs were formed and purified as described for the quench flow experiments, only in the absence of [^{32}P]-GTP. The purified complexes were split into two sample groups (Fig. S6). To the preincubation sample group, 10 μM [α-^{32}P]-ATP was added to the SECs prior to dividing the reaction into four aliquots of equal volume. The preincubation reactions were initiated with the following NTP mixtures: 100 μM CTP; 100 μM CTP and 100 μM ATP; 100 μM CTP and 1 mM ATP; or 100 μM CTP and 5 mM ATP. The simultaneous addition sample group reactions were initiated with the same NTP mixtures except that 10 μM [α-^{32}P]-ATP (800 Ci/mmol) was also included in all the mixtures. Reactions were allowed to proceed for ∼10 s before quenching with 0.5 M EDTA. Samples were processed the same as the EDTA rapid quench flow experiments.

Data analysis. The percentage of complexes at each position was determined by dividing the amount of radioactivity of each band by the total amount of radioactivity in all the bands greater than or equal to 24 nucleotides in length [i.e., (band 24) + (band 25)+ (band 26) + ... = 100%] (plots in Fig. S1). Due to the observed misincorporation (i.e., complexes at template position +25 and higher) during SEC formation, it was necessary to normalize the data such that at 0 time, there was 0% incorporation and upon completion, there was 100% incorporation for CMP incorporation. To normalize the data for CMP incorporation, the sum of the percentage of complexes that were at +25 or higher [i.e., (band 25) + (band 26) + (band 27) + ...] prior to the addition of CTP were subtracted from each time point. The data were then normalized to 100% by dividing each time point by the highest percentage of complexes that reached +25 or higher. Normalization of the data from AMP incorporation was performed in a similar manner, with a few modifications: (*i*) the sum of the percentage of complexes that were at +26 and higher, instead of +25 and higher, [i.e., (band 26) + (band 27) + ...] prior to the addition of CTP were subtracted from each time point. (*ii*) AMP incorporation was normalized as a percentage of CMP incorporation by dividing each time point by the highest percentage of complexes that reached +25 or higher. The incorporation of AMP was normalized to the extent of CMP incorporation because AMP cannot incorporate without CMP being incorporated first. There are always some complexes stalled at +24 that do not elongate over the time course of the experiment, but do elongate in the chase reactions. In general, 70 to 80% of the complexes reach position +25 on the time scale of these reactions (1 s). As discussed previously, we consistently observe this extent of CMP incorporation and it is not dependent of the preparation of RNA polymerase (2, 3). The data were fit to single or double exponentials using the equations $(a + b * (1\text{-}\exp(-c * x))$ and $(a - b * \exp(-c * x) - (a - b) * (\exp(-d * x))$, respectively, where a, b, c, and d are variables.

1. Santangelo TJ, Mooney RA, Landick R, Roberts JW (2003) RNA polymerase mutations that impair conversion to a termination-resistant complex by Q antiterminator proteins. *Genes Dev* 17:1281–1292.
2. Foster JE, Holmes SF, Erie DA (2001) Allosteric binding of nucleoside triphosphates to RNA polymerase regulates transcription elongation. *Cell* 106:243–252.
3. Holmes SF, Santangelo TJ, Cunningham CK, Roberts JW, Erie DA (2006) Kinetic investigation of *Escherichia coli* RNA polymerase mutants that influence nucleotide discrimination and transcription fidelity. *J Biol Chem* 281:18677–18683.

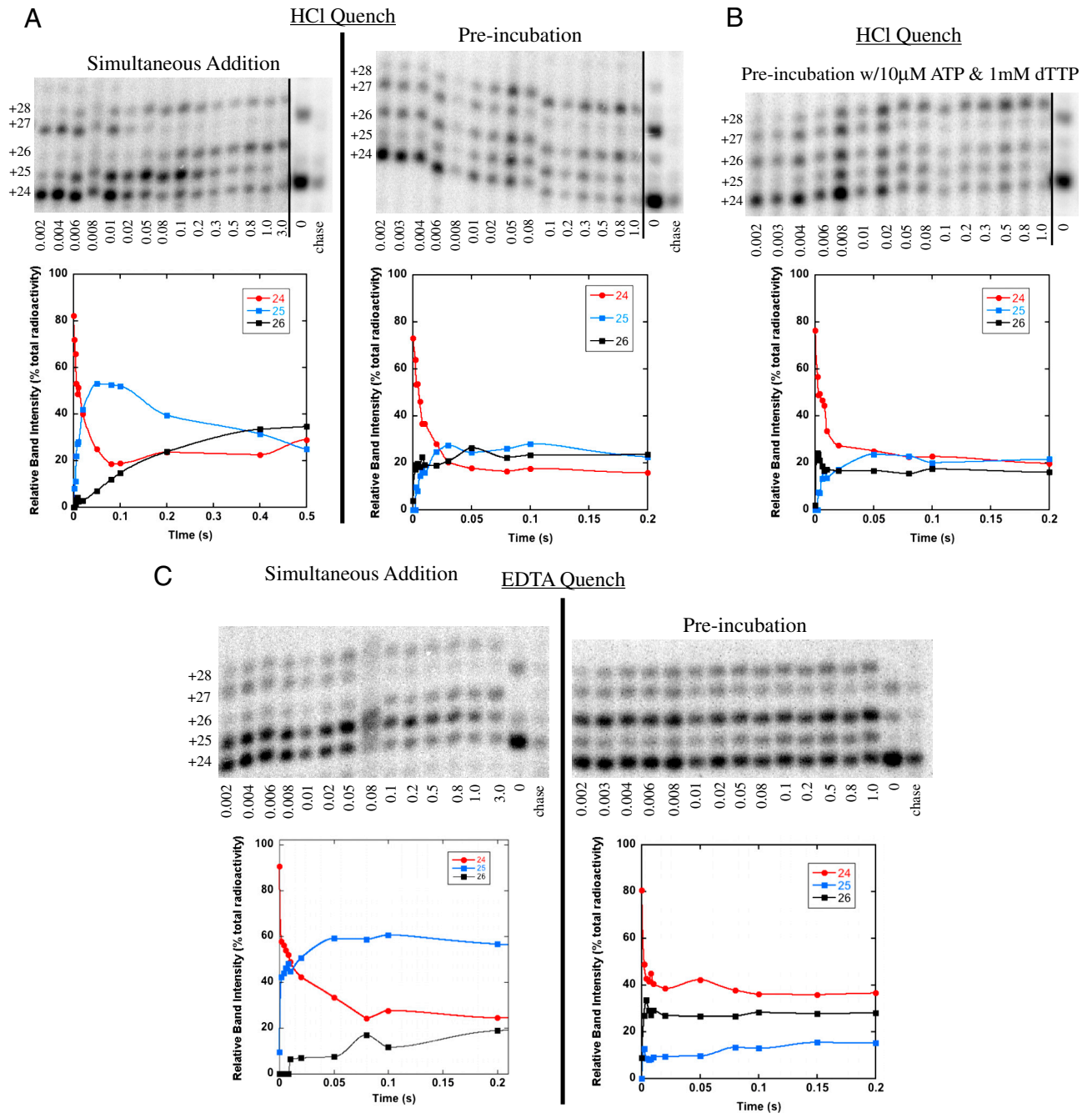

Fig. S1. Representative gels and their respective plots of the relative band intensities at each time point for template positions +24 (red circles), +25 (blue squares), and +26 (black squares) for (*A*) the simultaneous and preincubation experiments quenched with 1 M HCl, (*B*) the preincubation experiment with 10 μM ATP and 1 mM dTTP quenched with HCl, and (*C*) simultaneous and preincubation experiments quenched with 0.5 M EDTA. (The plots show the relative intensities of each band and not the sum of intensities of bands of a given length and longer.) Note that in the preincubation experiments, the plots indicate that band at +26 appears before the band at +25; whereas, in the simultaneous addition experiments, the band at +25 appears before the band at +26. This observation indicates that AMP (band 26) is incorporated very rapidly after the incorporation of CMP relative to the simultaneous addition experiments, in which CMP incorporation is significantly faster than AMP incorporation, and there is an accumulation of CMP product. The contrast in the "0" and "chase" lanes in the gels in *A* and *B* were altered relative to the other lanes of the same experiment for clarity. The relative intensities of the bands within those lanes are unchanged. Approximately 20 to 30% of the complexes remain at position +24 over the time course of the reaction. This extent is typical and does not depend on the RNAP preparation (see *SI Methods* and references cited therein).

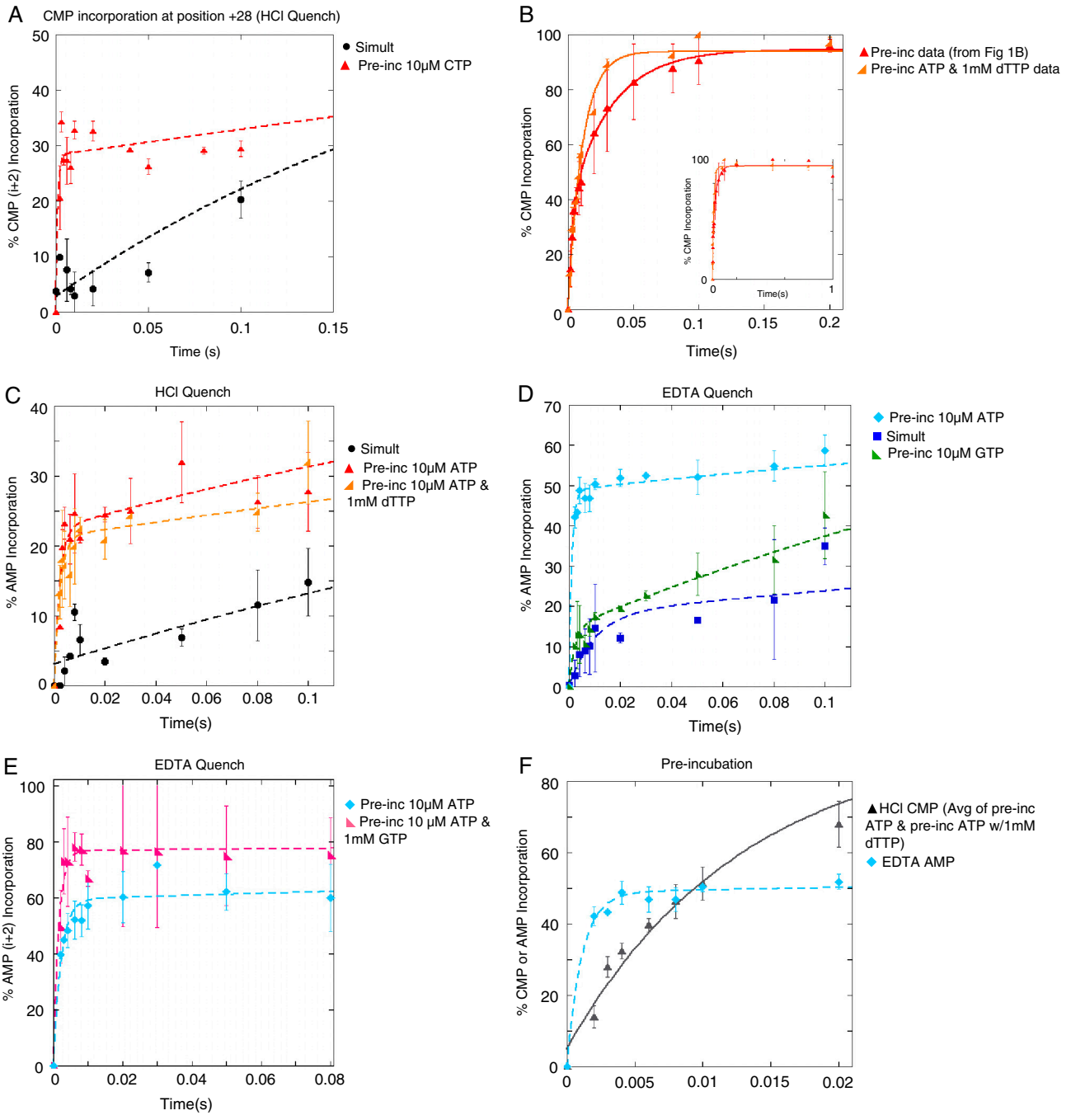

Fig. S2. The preincubation effect is specific for the $i + 2$ NTP. All data are the average of 2 to 4 experiments and the error bars show the standard deviation. The curves are fits to single or double exponentials. (*A*) Preincubation is independent of template base position and identity. For complexes stalled at position +26, plots of the percentage of complexes [normalized to GMP ($i + 1$)] that incorporated CMP ($i + 2$) at template position +28 for the simultaneous addition of 100 μM GTP and 10 μM CTP (black circles) and for preincubation with 10 μM CTP followed by initiation with 100 μM GTP (red triangles) when quenched with HCl are shown. Experiments were performed by walking SECs to template position +26 with 10 μM CTP and 10 μM ATP and purifying away the remaining NTPs. The SECs at +26 were then used in the indicated experiments. (*B*) Comparison of CMP ($i + 1$) incorporation for the preincubation of ATP alone (red triangles) (taken from Fig. 1*B*) and for the preincubation of ATP along with 1 mM dTTP (orange triangles) and quenched with HCl. (inset) Plot shows that there is no significant difference between these datasets over the course of the entire reaction (1 s). (*C*) Preincubation of SECs with a nonspecific NTP competitor along with ATP does not affect AMP incorporation. Plots of the percentage of complexes (normalized to CMP) that have incorporated AMP ($i + 2$) at template position +26 for the simultaneous addition of 10 μM ATP and 100 μM CTP (black circles), for the preincubation of 10 μM ATP followed by the addition of 100 μM CTP (red triangles), and for preincubation of 10 μM ATP along with 1 mM dTTP followed by the addition of 100 μM CTP (orange wedges) and quenched with HCl are shown. The data in the absence of dTTP are replotted from Fig. 1*C*. (*D*) Comparison of the percentage of apparent AMP incorporation for the preincubation of ATP and GTP using an EDTA quench. Plots of the percentage of complexes (normalized to CMP) that have apparently incorporated AMP ($i + 2$) at template position +26 for the simultaneous addition of 100 μM CTP and 10 μM ATP (blue squares), for the preincubation of 10 μM ATP followed by the addition of 100 μM CTP (cyan diamonds), and for the preincubation of 10 μM GTP followed by the addition of 100 μM CTP and 10 μM ATP (green wedges). The data for the preincubation of 10 μM ATP are replotted from Fig. 2*C*. (*E*) Plots of the percentage of complexes (normalized to CMP) that have incorporated AMP ($i + 2$) at template position +26 for the preincubation of 10 μM ATP followed by the addition of 100 μM CTP (red triangles) and for preincubation with 10 μM ATP and 1 mM GTP followed by the addition of 100 μM CTP (magenta wedges) and quenched with EDTA. There is a modest increase in the burst height for AMP in the presence of 1 mM GTP,

which could result from an increase in the rate of pyrophosphate release in the presence of this high concentration of GTP (see main text). An increase in pyrophosphate release would increase the burst height if dissociation of pyrophosphate in the absence of the next NTP (GTP) has a slower or similar rate as dissociation of the sequestered NTP. This result does *not* imply that both the $i + 2$ and $i + 3$ NTPs are binding because GTP is the $i + 2$ NTP with respect to AMP. (*F*) Replot of Fig. 3*A* for the ATP preincubation experiments, showing the comparison of the apparent incorporation of AMP (cyan diamonds) from the EDTA experiments to the incorporation of CMP (gray triangles) from the HCl experiments, using the average of the data from the preincubation ATP alone and the preincubation of ATP with 1 mM dTTP experiments (Fig. S2*B*). The preincubation experiments with dTTP are averaged with those in the absence of dTTP because dTTP shows no significant effect on CMP incorporation, especially at early time points (Fig. S2*B*). These data further confirm that ATP can be shuttled from the noncatalytic site to the catalytic site.

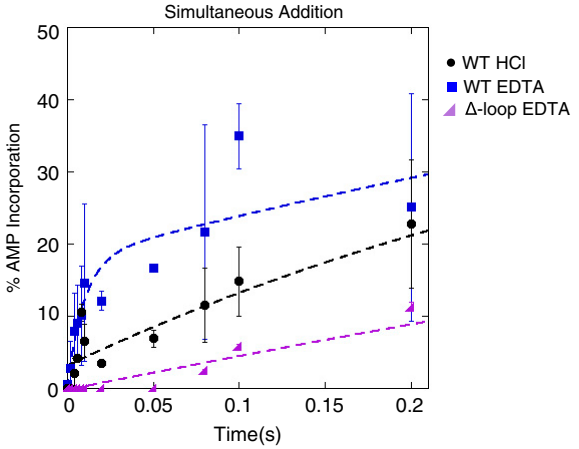

Fig. S3. Plots of the percentage of complexes (normalized to CMP) that have incorporated AMP ($i + 2$) at template position +26 for the simultaneous addition of 100 μM CTP and 10 μM ATP for wtRNAP quenched with HCl (black circles) or EDTA (blue squares) and for the Δ-loop mutant (purple wedges) quenched with EDTA. HCl data are replotted from Fig. 1*C*. Data are reported as an average of 2–4 independent experiments.

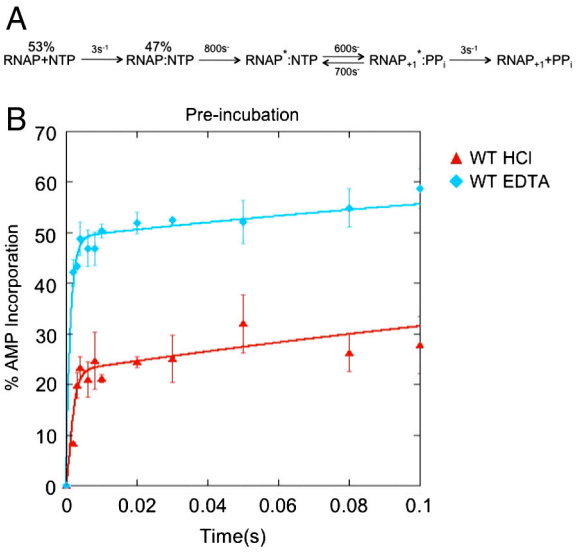

Fig. S4. Kinetic modeling of experimental data. (*A*) Reaction mechanism used to fit experimental data. (*B*) Plot of reaction mechanism with data from the preincubation experiments quenched with EDTA (blue diamonds) or HCl (red triangles). The curves were generated based on the mechanism shown in *A*. The data are replotted from Fig. 2*C*.

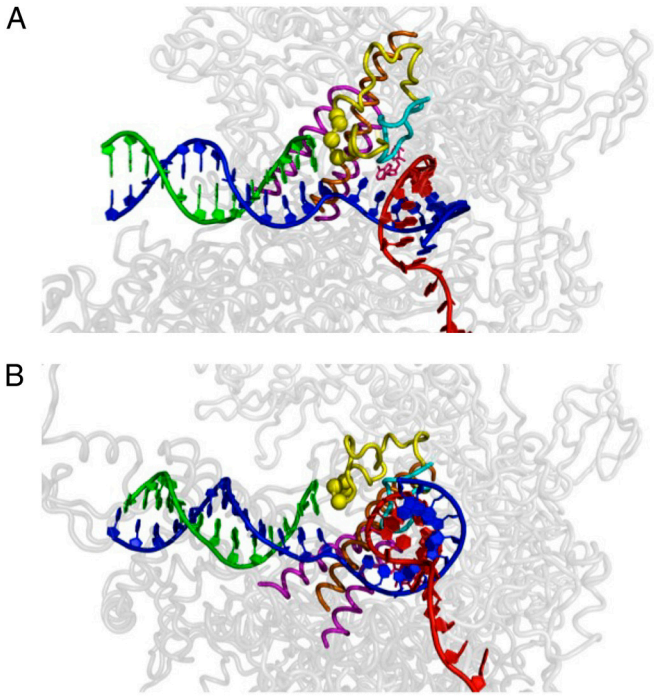

Fig. S5. Location of fork loop 2 in relation to other important structural elements. Shown are the (*A*) side and (*B*) top views of the β and β′-subunits (transparent gray) of the *Thermus aquaticus* transcription elongation complex (PDB: 2O5J). Several key structural elements are highlighted. The nontemplate and template DNA strands are highlighted in green and blue, respectively. The template and nontemplate DNA are separated at fork loop 2 (yellow). The rifampicin binding region of fork loop 2 is cyan. The nascent RNA chain is red. The catalytically essential trigger loop (magenta) is located below the bridge helix (orange) and is shown interacting with a nucleotide (pink) in the catalytic site. The locations of the four deleted amino acids in fork loop 2 are denoted by the yellow spheres.

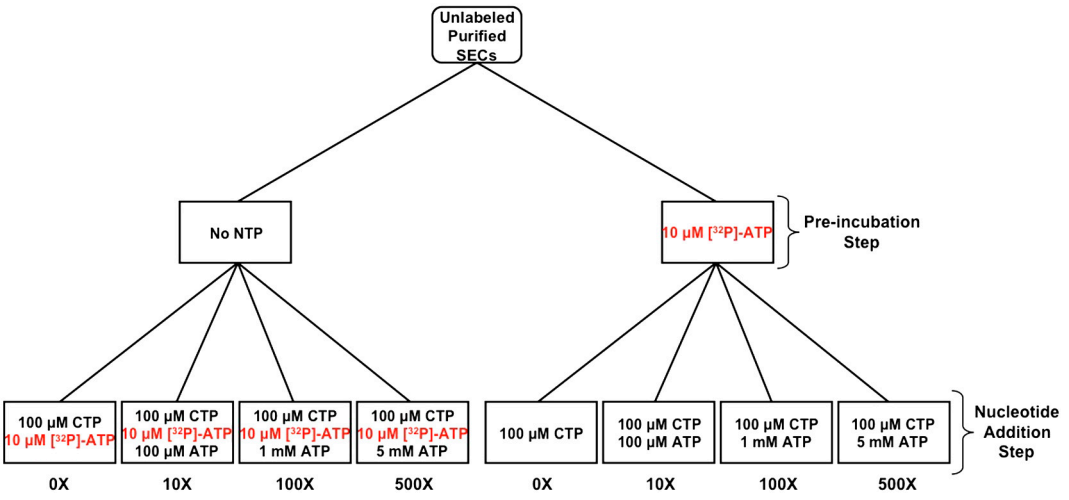

Fig. S6. Schematic outlining experiments to test if ATP can be shuttled from the noncatalytic site to the catalytic site.

Table S1. Kinetic parameters of CMP and AMP incorporation for wild-type RNAP

	Simultaneous			Preincubation		
	$k_{fast}(s^{-1})$*	k_{fast} Burst Height (%)	$k_{slow}(s^{-1})^{\dagger}$	$k_{fast}(s^{-1})$	k_{fast} Burst Height (%)	$k_{slow}(s^{-1})$
CMP HCl	-	-	43.9 ± 3.9	280 ± 112	36.0 ± 10.2	31.2 ± 7.7
AMP HCl	-	-	2.4 ± 0.3	466 ± 160	22.4 ± 2.0	3.0 ± 1.2
CMP EDTA	1,400[‡]	40.5 ± 2.6	43.4 ± 6.3	$1{,}550 \pm 852$	64.1 ± 2.5	15.4 ± 4.5
AMP EDTA	101 ± 42	17.8 ± 2.9	1.2 ± 0.3	902 ± 135	48.8 ± 1.0	4.2 ± 1.2

*k_{fast} is the rate of incorporation for the burst phase.
[†]k_{slow} is the rate of the slow phase observed in reactions exhibiting biphasic kinetics or is the rate constant for reactions fitting to a single exponential.
[‡]Rate was derived from a manual fit to the data for the fast phase.