Supplementary Materials

Yeast mtDNA transcription initiation in single nucleotide addition steps

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Movie legends

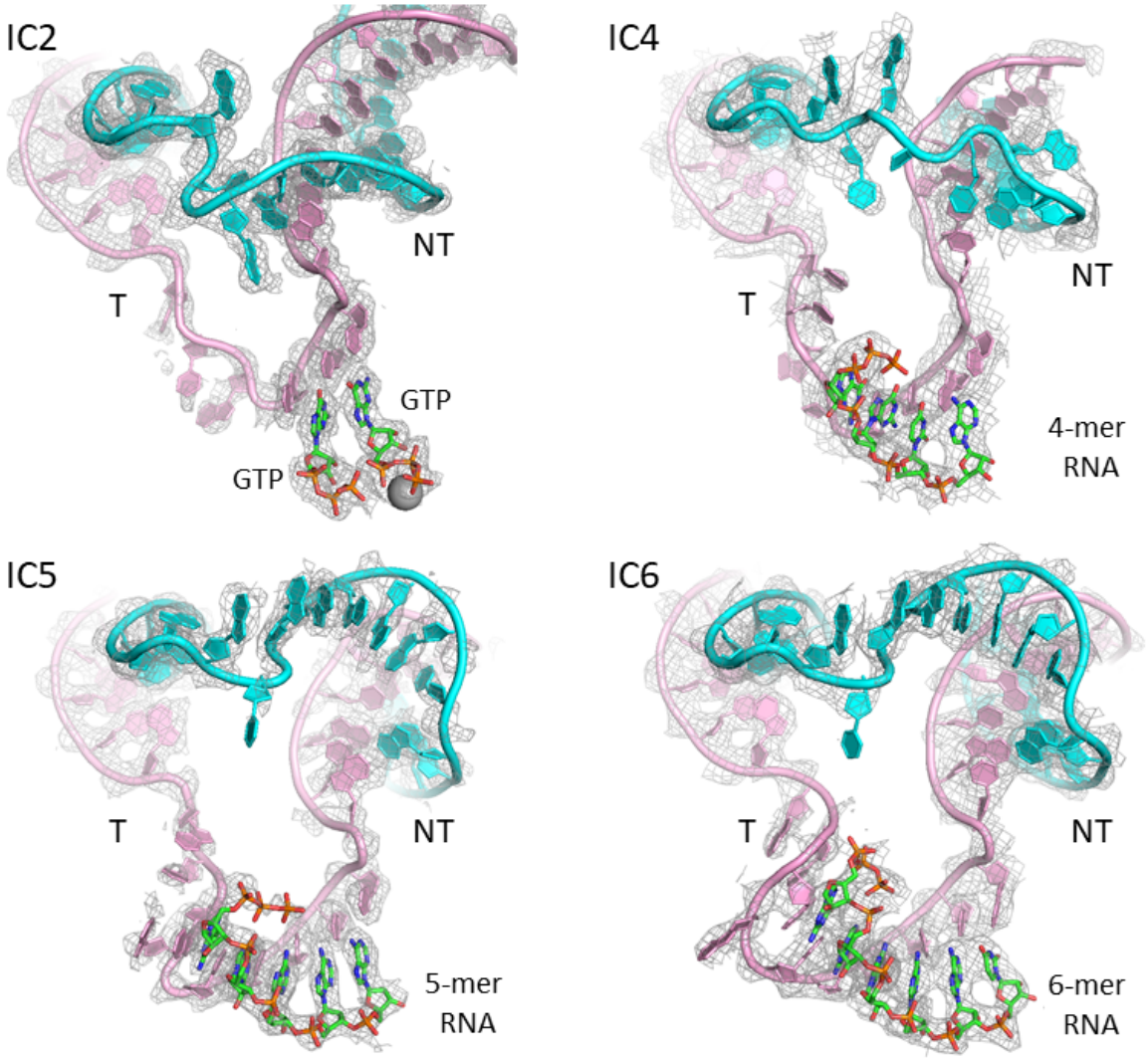
Movie 1. The structural changes of the nucleic acid parts showing the transition from PmIC to IC6 at single nucleotide addition steps and then from IC6 to EC. Morphing between the partially-melted initiation state (PmIC), initiation states (IC2 – IC6), and the elongation state (EC) simulates the conformational changes in promoter and proteins in the initiation complexes and during the transition to the elongation complex. The y-mtRNAP is in blue, MTF1 in yellow, non-template (NT) DNA in cyan, template DNA in pink, incoming NTPs in blue, incorporated RNA in yellow, template position -1 in green, and the template in red for the end states and the structural elements are in gray for the start states in the movie. The transcription bubble opens fully during the transition from PmIC to IC2; the +1 and +2 bases flip towards the polymerase active site and base-pair with the incoming NTPs to initiate *de novo* RNA synthesis. This promoter melting is accompanied by further bending of the downstream DNA by about 60° with respect to the upstream DNA. The promoter DNA maintains stable upstream DNA interactions with the y-mtRNAP and MTF1 and is spatially confined throughout the initiation steps PmIC → IC6. In the PmIC → IC2 transition, the transcription bubble fully opens, and the downstream template bases are brought in to align with two initiating GTP molecules at the active site; this transition is accompanied by the non-template (NT) scrunching and the single-strand part of the template taking a "U" shape. In the IC2 → IC3 transition: (i) the third nucleotide (an UTPαS) binds at the N site and is poised for catalytic incorporation, (ii) the template starts to bulge as the RNA:DNA duplex pushes the single strand template region of -4 to -1 nucleotides, and (iii) the NT loop scrunches further. In the IC3 → IC4 transition: (i) the 4^{th} nucleotide is incorporated to the RNA strand, (ii) the template bulging expands, however, the stacking of -1 template base (in green) with the RNA:DNA is dissociated, and (iii) the scrunched NT strand is liberated. The single strand parts of the template and non-template in the transcription bubble are significantly less ordered and lack interactions with protein residues. In the IC4 → IC5 transition: (i) the 5-mer RNA is formed, (ii) the template -2 base is stacked with RNA:DNA on one side and with -1 base on the other side, (iii) surprisingly the NT strand switches its position and conformation to stack +1 to +6 nucleotide bases in an orderly fashion as a "staircase-like" structure, (iv) the +6 base pair is melted from the downstream duplex and the +6 NT base is engaged in the stable NT base-stacked structure, whereas, the +6 template base is ready to enter the polymerase cleft for binding of the next NTP. In the IC5 → IC6 transition, (i) the template is translocated by one nucleotide while the NT strand has changed a little, (ii) RNA grows to a 6-mer, and (iii) the template -1 adenine base has moved significantly to occupy a pocket adjacent to the thumb subdomain. We obtained IC6

as the last stable state before the complex enters EC; our attempt to trap IC7 resulted in IC6 only. The large conformational changes in the transition from IC6 → EC are visualized by morphing a y-mtRNAP:RNA:DNA EC structure, modeled based on h-mtRNAP:RNA:DNA EC structure (PDB ID. 4BOC), with the y-mtRNAP IC6 structure. The RNA:DNA and downstream DNA duplexes in both structures superimpose, whereas the upstream DNA is released from its interactions with MTF1 and y-mtRNAP and switches conformation to enter the elongation phase. In the process, MTF1 is released, and the -4 to -1 bases of the template and NT reanneal. The above structural transitions are shown as a single uninterpreted clip in the end.

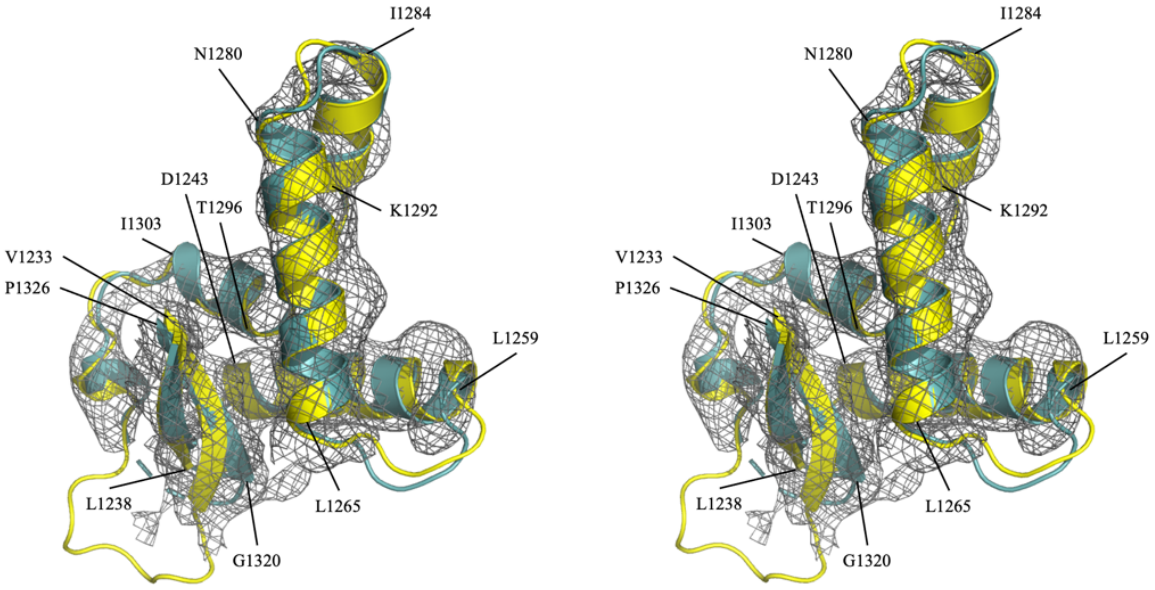
Movie 2. The structural changes of y-mtRNAP and MTF1 through the transition from PmIC to IC6 at single nucleotide addition steps and then from IC6 to EC. The PmIC → IC2 transition is associated with the sliding of MTF1 over y-mtRNAP to close the polymerase and downstream clefts for holding the downstream DNA that helps the bubble fully open and engage the incoming NTPs for *de novo* initiation. The thumb subdomain also moves with MTF1 maintaining the protein:protein interactions. The clefts gradually open with each nucleotide addition to accommodate the growing transcription bubble, and in IC4 the relative positioning of MTF1 and y-mtRNAP almost returns to that in PmIC. In the IC5 and IC6 states, the MTF1 moves in a lateral direction with respect to y-mtRNAP, which is ~90º from its closing/opening movements in the PmIC to IC4 transitions (SI Fig. S3). The direction of the movement in IC5 and IC6 appears to be along a path that helps the release of MTF1 between the IC6 → EC transition over the next few nucleotide additions. The IC6 → EC transition is expected to associate with large conformational changes of the upstream DNA, release of MTF1, and switching in the role of the MTF1-hairpin that holds the C-terminal domain of MTF1 in IC states to accommodate the upstream DNA in EC. The C-tail in the polymerase cleft plays an important role and undergoes large positional and conformational changes to stabilize the transcription bubble at different IC states.

Supplementary Table 1. Single Particle Cryo-EM Data and Structure Analysis Statistics				
Structure	IC2 (y-mtRNAP; MTF1; dsDNA; 2GTP)	IC4 (y-mtRNAP; MTF1; dsDNA; pppGpGpUpA)	IC5 (y-mtRNAP; MTF1; dsDNA; pppGpGpApApA)	IC6 (y-mtRNAP; MTF1; dsDNA; pppGpGpApApApU)
PDB ID/EMBD ID	8AP1/EMD-15556	8ATT/EMD-15662	8ATV/EMD-15664	8ATW/EMD-15665
Data Collection				
Data collection date	18-12-2020	15-02-2022	24-02-2021	18-02-2022
Grid type	Quantifoil R 1.2/1.3 Cu300	Quantifoil R1.2/1.3 Au300	Quantifoil R 1.2/1.3 Cu300	Quantifoil R1.2/1.3 Au300
Number of grids	1	1	1	1
Microscope/detector	Glacios™ Cryo-TEM / Falcon 3	Glacios™ Cryo-TEM / Falcon 3	Glacios™ Cryo-TEM / Falcon 3	Glacios™ Cryo-TEM / Falcon 3
Voltage (kV)	200	200	200	200
Magnification	190,000x	150,000x	150,000x	150,000x
Recording mode	Counting	Counting	Counting	Counting
Dose (e/Å^2/frame)	1.58	1.04	1.04	1.04
Total dose (e/Å^2)	40	40	40	40
Number of frames/movies	40	40	40	40
Total exposure time (s)	25.15	38.41	38.41	38.41
Pixel size (Å)	0.76	0.97	0.97	0.97
Defocus range (Å)	-8,000 to -22,000	-8,000 to -18,000	-8,000 to -22,000	-8,000 to -18,000
Data processing				
Number of micrographs used	2,045	997	602	1,627
Number of particles picked	1,149,870	671,969	549,988	940,973
Number of particles after 2D/3D clean	309,765	296,664	192,599	275,877
Particles used for final map	137,631	138,730	91,298	144,092
Map resolution (FSC 0.143; Å)	3.47	3.44	3.39	3.62
Map sharpening B factor (Å^2)	-144	-80	-140	-125
Model fitting				
Experimental map/model correlation	0.82	0.72	0.77	0.79
Total number of atoms	11,229	11,153	11,161	11,283
Average B factor (Å^2)				
Protein atoms	22.34	88.32	28.60	75.27
Nucleid acid and NTP	51.85	80.43	79.50	95.37
Clash score	5.85	9.84	6.95	8.42
Ramachandran plot; favored/outlier (%)	96.07/0.00	97.58/0.00	96.64/0.00	97.20/0.00
Rotamer outlier (%)	0.27	0.18	0.00	0.00
RMSD bond length (Å)/bond angle (°)	0.005/0.762	0.006/1.080	0.005/1.008	0.006/1.023

Supplementary Figures

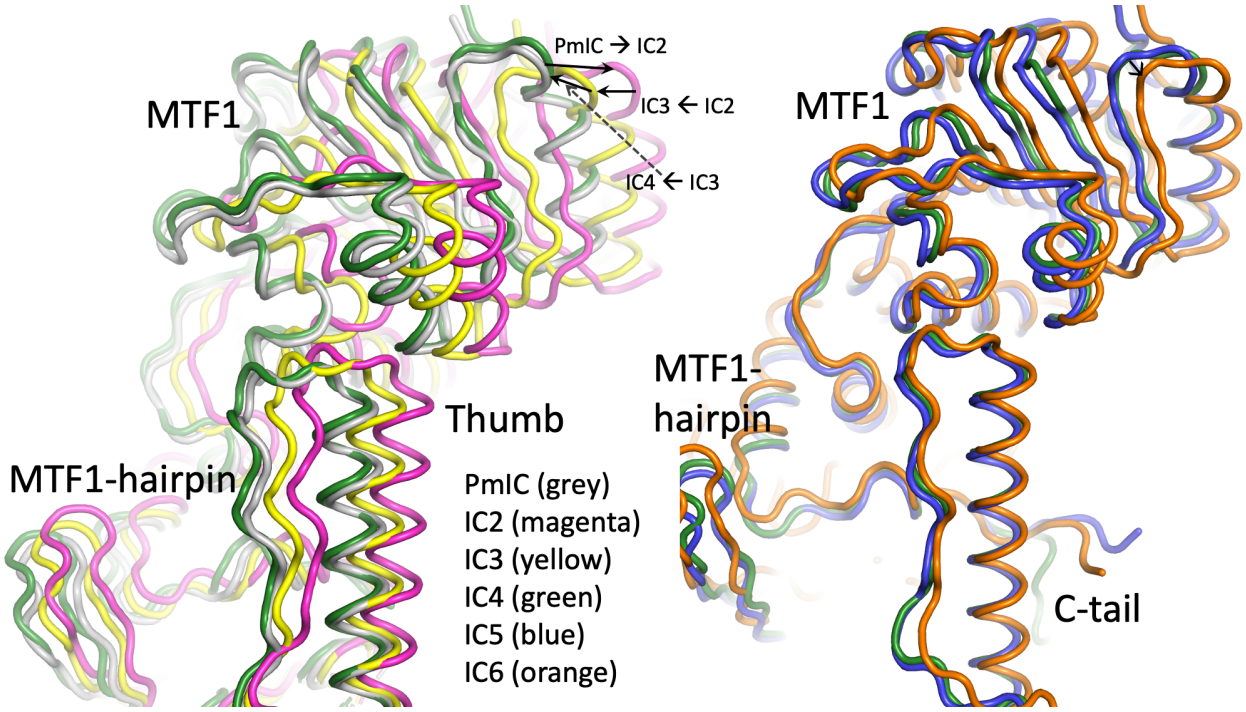


Supplementary Fig. 1. Density maps of the transcription bubbles in IC2, IC4, IC5 and IC6. The density maps clearly define the position and conformation of complete transcription bubbles in different IC structures; with the non-template (NT) in cyan, template (T) in pink, and GTP and RNA in green C-atom representation. The contour levels of the density maps are 1.75, 1.3, 2, and 2σ for IC2, IC4, IC5 and IC6, respectively. The shown density map for IC4 is the unsharpened map as the B-sharpened map is noise. Remaining three maps are B-sharpened.

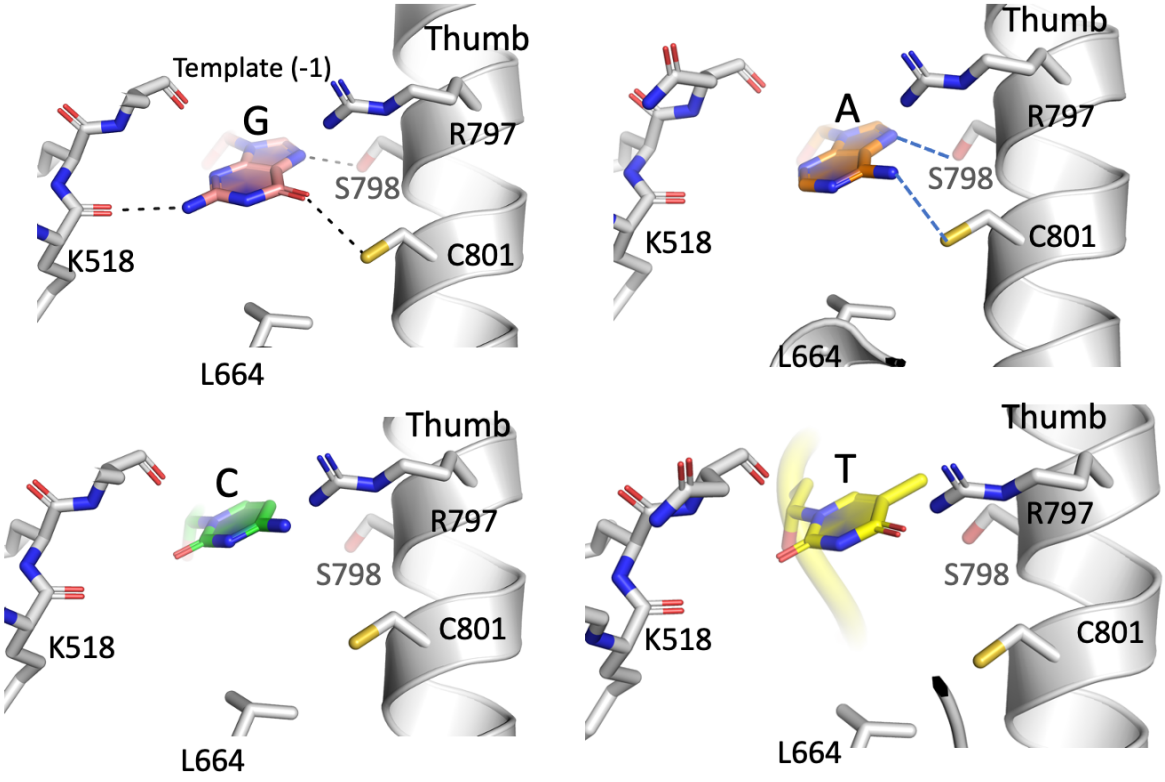


Supplementary Fig. 2. Stereo view of y-ins region.

Wall-eyed stereo view showing the starting y-ins AlphaFold model (UniProt ID: P13433 - yellow) that was fitted to the density. The final model (turquoise) after density fit also aligns well with the AlphaFold model; the density segment for the y-ins domain in the IC6 structure is contoured at 2.5σ.

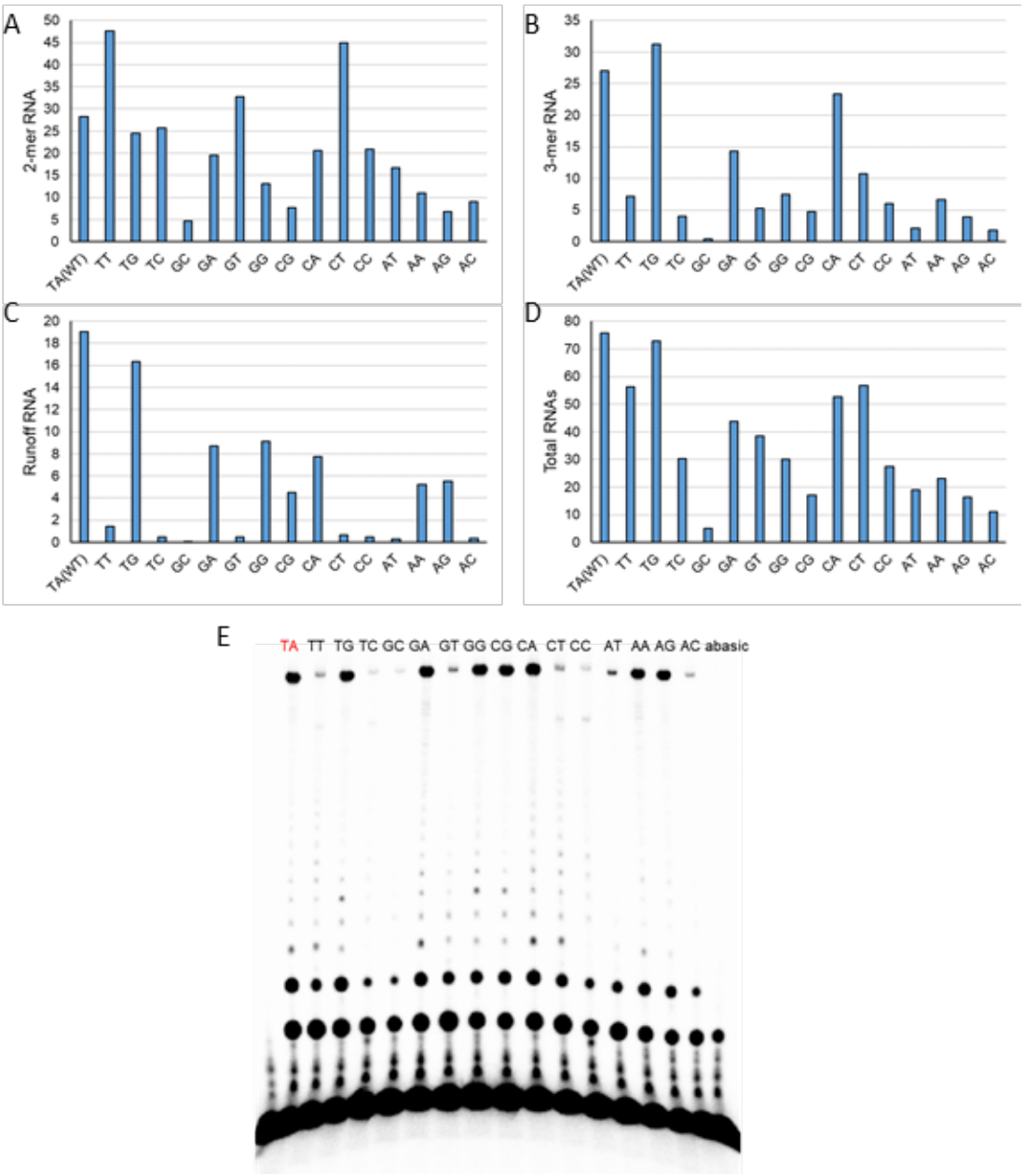


Supplementary Fig. 3. Superposition of MTF1 in different IC states.
Positioning of MTF1 and y-mtRNAP (thumb and MTF1-hairpin) in different IC states; from PmIC to IC4 (left) and IC4 to IC6 (right). The structures were aligned based on y-mtRNAP superposition.

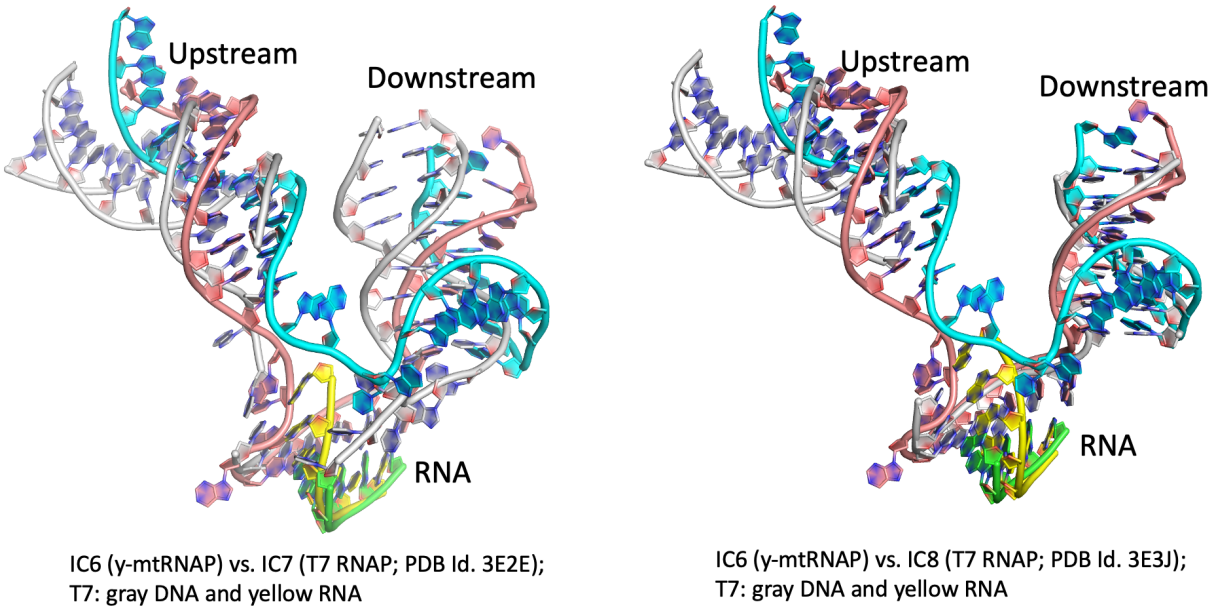


Supplementary Fig. 4. Modeling of A/C/T bases into the template -1 pocket in IC6 structure.

Potential interactions of template -1 nucleotide in IC6 structure when a guanine (G) is substituted with adenine (A), cytosine (C), and thymine (T). The base interactions are significantly reduced by a purine to pyrimidine substitution.



Supplementary Fig. 5. In vitro transcription on -1 position modified promoters.
The quantitively analyses of the gel in Fig. 4G show the effect of -1 position changes on 2-mer (A), 3-mer (B), runoff (C), and total RNA synthesis (D). The gel image from a repeated experiment demonstrating reproducibility of the data. An abasic -1 template position was included in this repeat (E).

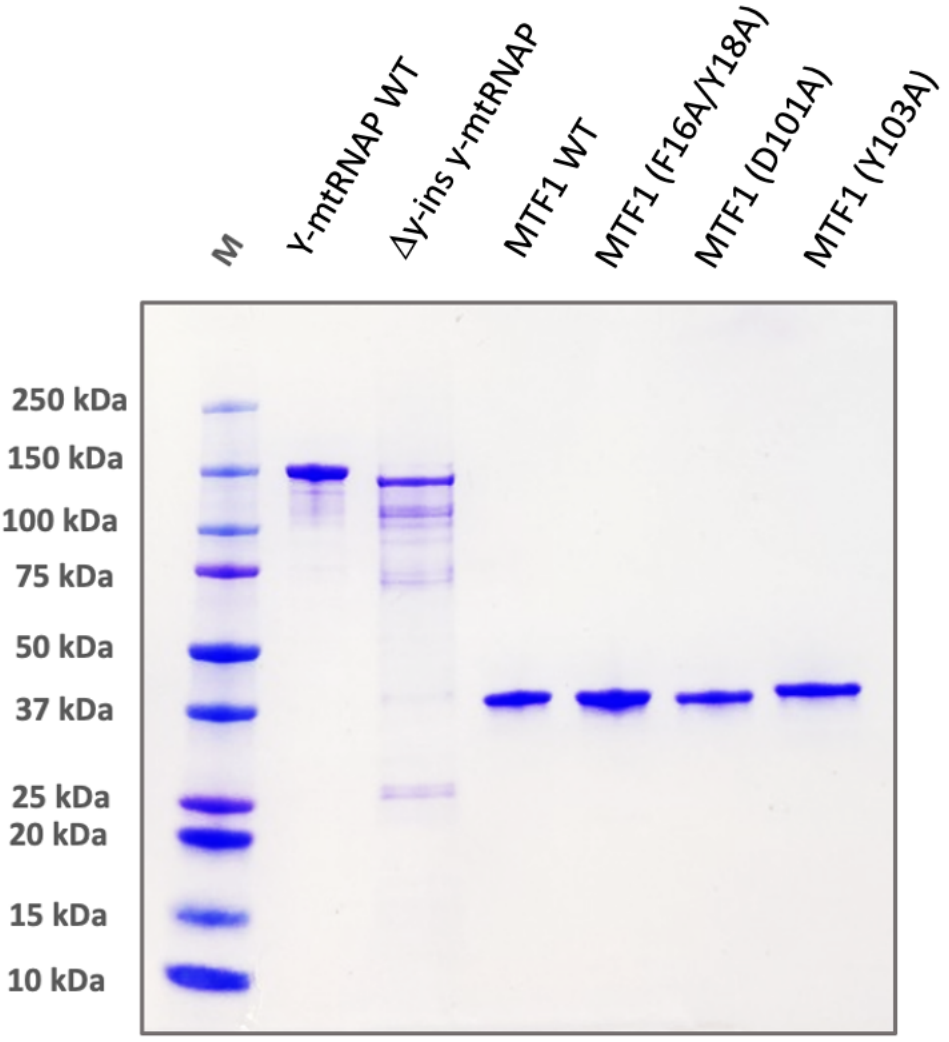


IC6 (y-mtRNAP) vs. IC7 (T7 RNAP; PDB Id. 3E2E); T7: gray DNA and yellow RNA

IC6 (y-mtRNAP) vs. IC8 (T7 RNAP; PDB Id. 3E3J); T7: gray DNA and yellow RNA

Supplementary Fig. 6. Superposition of IC6 on T7 RNAP IC7 and IC8.

Relative positions of upstream and downstream DNA, and RNA:DNA duplex in y-mtRNAP IC6 with T7 RNAP IC7 (left) and IC8 (right). The template, non-template, and RNA in IC6 are colored pink, cyan, and green, respectively. The DNA and RNA in T7 IC7 and IC8 are colored gray and yellow, respectively. The alignments were based on RNAP Cα superposition.



Supplementary Fig. 7. SDS-PAGE analysis of different y-mtRNAP and MTF1 proteins. Purified wild-type and mutant proteins (y-mtRNAP and MTF1) used in transcription runoff assays.