Supplementary Materials for

Replication-transcription switch in human mitochondria

Karen Agaronyan, Yaroslav I. Morozov, Michael Anikin, and Dmitry Temiakov*

Department of Cell Biology, Rowan University, School of Osteopathic Medicine, 2 Medical Center Dr, Stratford, NJ 08084,USA.

*Corresponding author. E-mail: d.temiakov@rowan.edu

This PDF file includes:

Material and Methods

Proteins.

Human mtRNAP, TFAM and TFB2M were purified as described previously (*4*). C-terminal His-6 TEFM (mature form, residues 36-360) was cloned into pET22b vector and expressed in BLR (DE3, recA-) cells (Novagen). The overnight cell culture (5 ml) was used to inoculate 1L of LB and the cell culture was incubated for 3-4 h at 37^0C until OD at 600 nm reached 0.4 units. The flasks were then transferred to 16^0C and incubated for additional 40 min prior to addition of IPTG (0.2 mM). The cells were harvested after 18 h of incubation and disrupted by sonication. TEFM was first purified by affinity chromatography on Ni-agarose beads (Qiagen) followed by heparin-sepharose purification in 250-1500 mM gradient of NaCl. The protein was concentrated to 3-10 μM concentration, diluted 2 times with glycerol, aliquoted and stored at -70^0C.

Transcription assays.

Templates for the transcription assays involving CSBII were amplified by PCR from a plasmid containing region 202-481 of human mtDNA. Note that the reference human mtDNA (Cambridge) contains a rare polymorphism in the CSBII region (311-315) (*14*) and therefore a more commonly occurring in humans CSBII sequence (G6AG8) was used in this study. A variant of a PCR template ("del71 CSBII") with the deletion of 71 bps region between the LSP promoter and CSBII (bps 388-318) was used for transcription and cross-linking assays. To prepare templates producing 500 nt or 1000 nt run-off products we PCR-amplified regions of pT7blue plasmid containing -70 to + 70 region of the LSP promoter (region 339-478 in human mtDNA). Standard transcription reactions were carried out as described previously (*4*) using

DNA templates (50 nM), mtRNAP (150 nM), TFAM (200 nM), TFB2M (150 nM), TEFM (300 nM) in a transcription buffer containing 40 mM Tris (pH=7.9), 10 mM $MgCl_2$ and 10 mM DTT in the presence of ATP (0.3 mM), GTP (0.3 mM), CTP (0.3 mM), UTP (0.01 mM) and 0.3 μCi [α-^{32}P] UTP (800 Ci/mmol). Reactions were carried out at 35^{0}C for the time indicated in figure legends and stopped by addition of an equal volume of 95% formamide/ 0.05M EDTA. The products were resolved by 20% PAGE containing 6 M urea and visualized by PhosphoImager (GE Health). Nucleic acid scaffold complexes were assembled as described (*15*).

RNA-protein and DNA-protein cross-linking.

To probe interactions between TEFM and RNA in a scaffold EC, the photo cross-linking probe 4-thio UMP was incorporated into 15 nt RNA oligo (5'-A/4-thioU/GUCUGCGGCGCGC, Dharmacon). DNA-TEFM cross-linking was probed using DNA oligos (Midland Scientific) with 4-thio dTMP incorporated into the template DNA strand (3' GTACCCCATCGCCGCGCGTGCGG/4-thio-dT/CTGC-5') or into the non-template DNA strand (5'- CATGGGGTATTTATTT/4-thio-dT/GACGCCAGACG-3'). The non-modified oligos used to prepare scaffolds were as described previously (15). Note that the positions of the probes relative to the 3' end of the RNA are given according to the crystal structure of mtRNAP EC (15). The photo-reactive oligos were 5' ^{32}P-radiolabeled and the scaffolds were annealed as described previously (*15*). The ECs were assembled using 1 μM scaffold, mtRNAP (1μM) and TEFM (2 μM) for 10 min and UV-irradiated for 10 min at room temperature. The cross-linked species were resolved using 4-12% SDS PAGE (Invitrogen) and visualized by PhosphoImager (GE Health). To probe interactions of TEFM with the nascent RNA that encompasses CSBII, the promoter PCR template (del71CSBII, see above) was used. The mtRNAP was allowed to make a

start-up complex and "walked" along the DNA to incorporate a photo reactive analog of GMP, 6-thio GMP (Axxora), as illustrated in Fig. S2. The cross-linking was performed as described above.

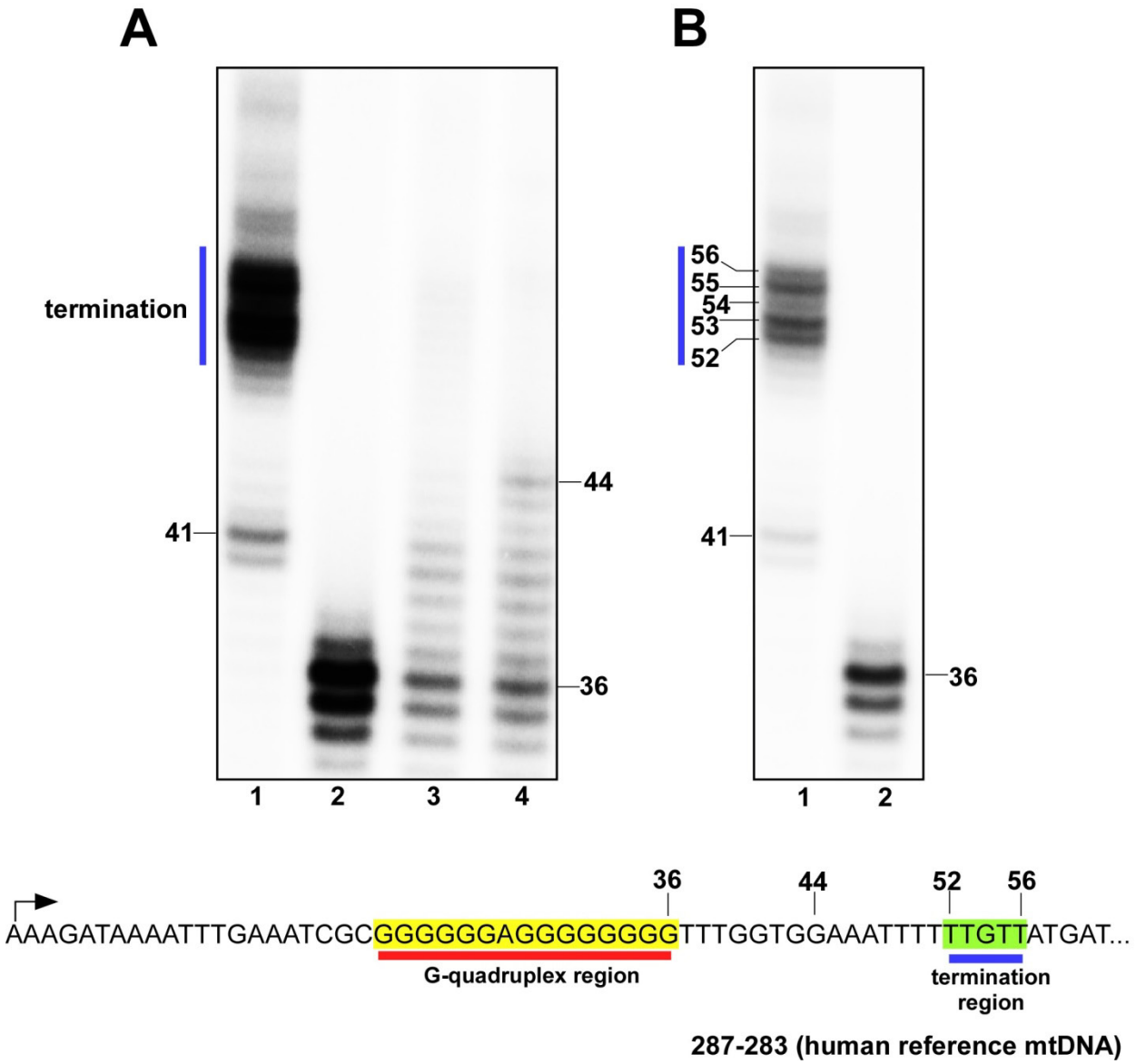
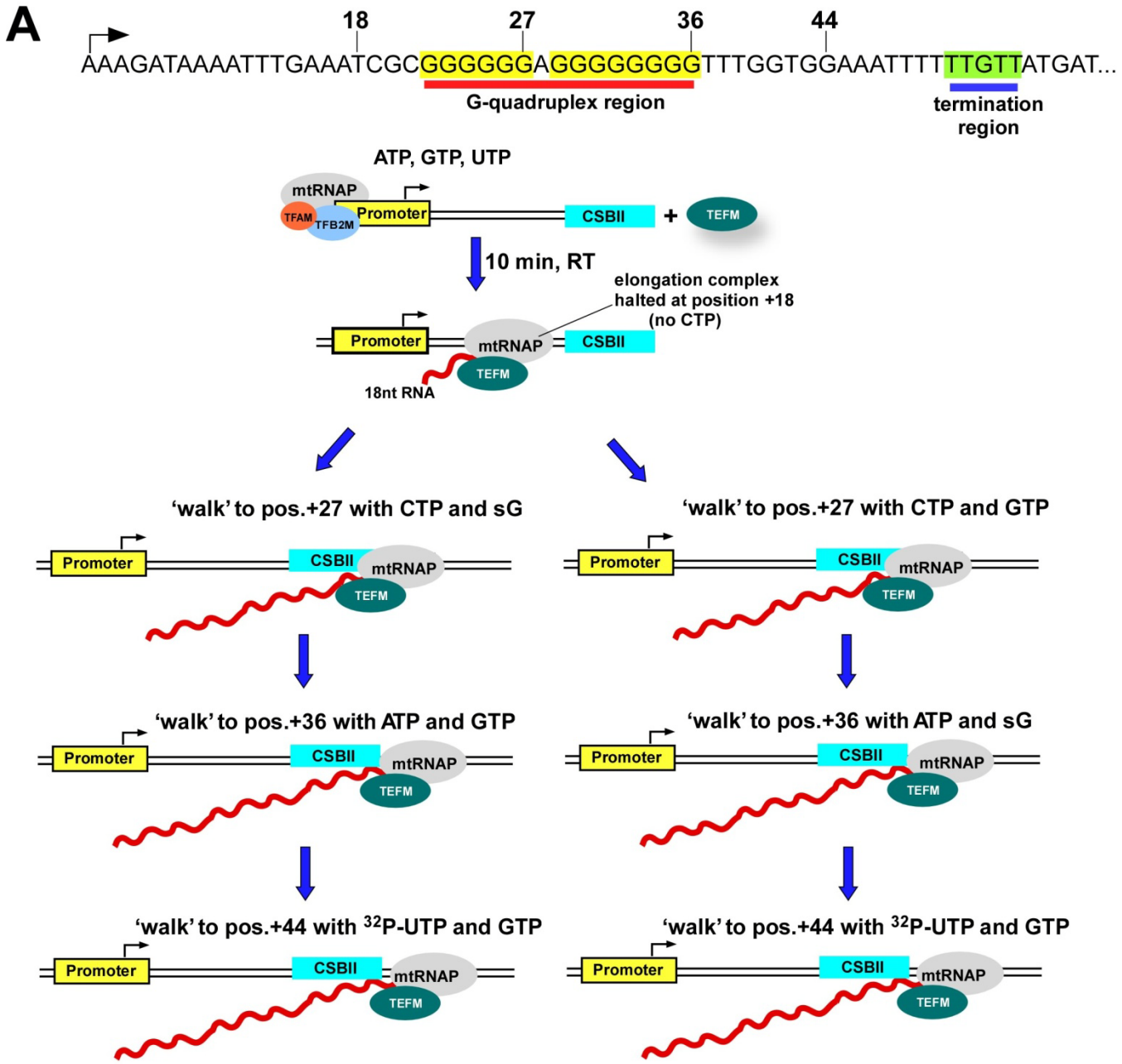


Figure S1. Mapping of the termination site at CSBII. (**A**) Transcription reactions were performed using del71CSBII template (the initial transcribed sequence is indicated below the panel). In the absence of TEFM, mtRNAP efficiently terminates at CSBII (lane 1). RNA markers (36 nt and 44 nt, lanes 2-4) were prepared by walking mtRNAP using Ni-agarose beads as described below in the Fig S2. (**B**) Lighter exposure of the autoradiogram shown in **A** (lane 1 and 2 only).


A
18
27
36
44
AAAGATAAAATTTGAAATCGCGGGGGGAGGGGGGGGTTTGGTGGAAATTTTTTGTTATGAT...
G-quadruplex region
termination region
ATP, GTP, UTP
mtRNAP
TFAM
TFB2M
Promoter
CSBII
+
TEFM
10 min, RT
elongation complex halted at position +18 (no CTP)
Promoter
mtRNAP
CSBII
TEFM
18nt RNA
'walk' to pos.+27 with CTP and sG
'walk' to pos.+27 with CTP and GTP
'walk' to pos.+36 with ATP and GTP
'walk' to pos.+36 with ATP and sG
'walk' to pos.+44 with 32P-UTP and GTP
'walk' to pos.+44 with 32P-UTP and GTP

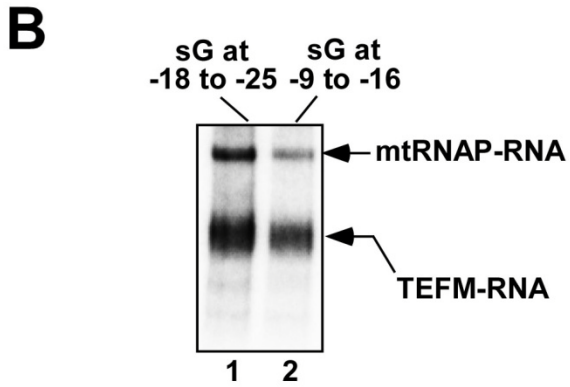

B
sG at -18 to -25
sG at -9 to -16
mtRNAP-RNA
TEFM-RNA
1
2

Figure S2. TEFM interacts with the 5' end of the nascent transcript containing CSBII.

A. Incorporation of a photo reactive 6-thio-GMP into RNA transcript. The start-up complexes were formed on del71CSB2 template (the transcribed sequence is indicated at the top) by providing ATP (0.3 mM), GTP (0.3 mM), and UTP (0.3 mM) to allow mtRNAP to synthesize a 18-mer RNA in the presence of TEFM. These complexes were immobilized on Ni-agarose beads and washed with transcription buffer to remove substrate NTPs. The photo reactive analog, 6-thio GMP (sG, 50 μM) was incorporated during the subsequent steps, as indicated, and the ECs were halted 44 bps away from the promoter start site.

B. TEFM interacts with the RNA. The EC44 obtained as described above were UV-irradiated, eluted from Ni-agarose with 0.25 mM Imidazol and analyzed using 4-12% SDS-PAGE followed by autoradiography.

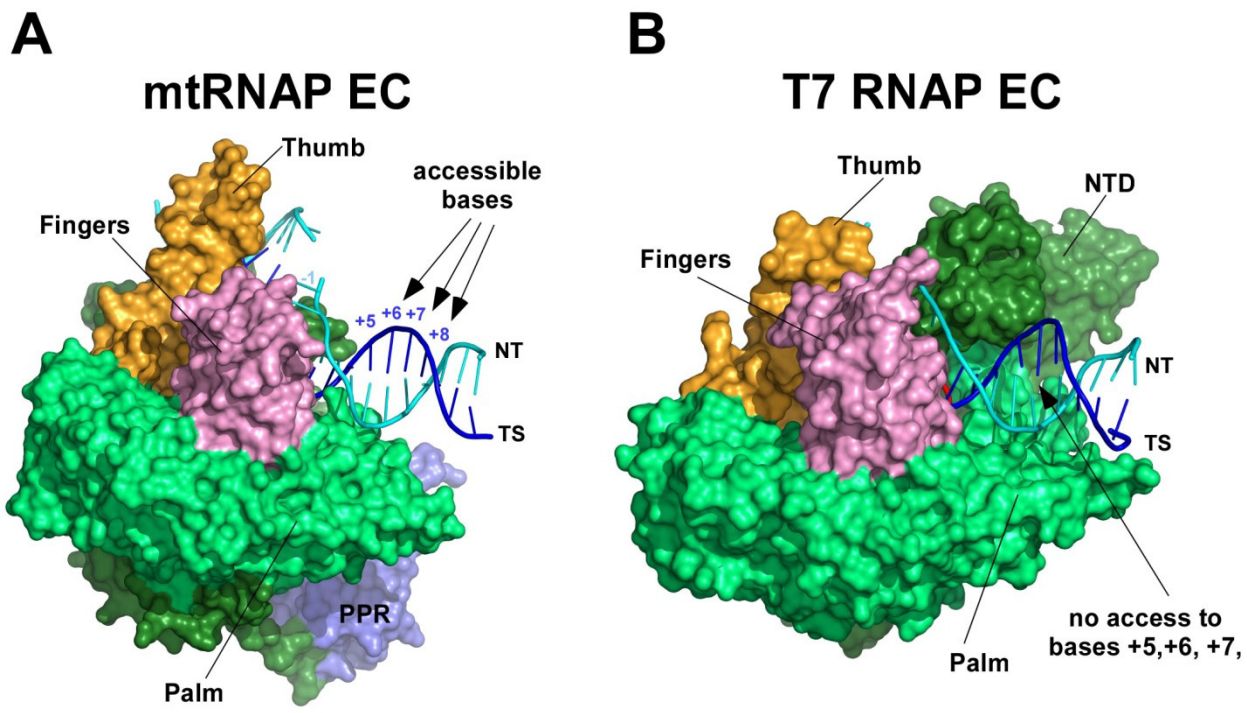


Figure S3. Downstream DNA region in mtRNAP EC is accessible for interactions with TEFM. (**A, B**) Elongation complexes of mtRNAP (PDB ID 3BOC) and T7 RNAP (PDB ID 1SVO) are aligned with respect to their conserved palm subdomains. Color code: fingers - pink, palm - light green, N-terminal domain - dark green, thumb - orange, PPR domain (mtRNAP) - light blue, DNA template strand (TS) - blue, non-template strand (NT) - cyan, RNA - red.

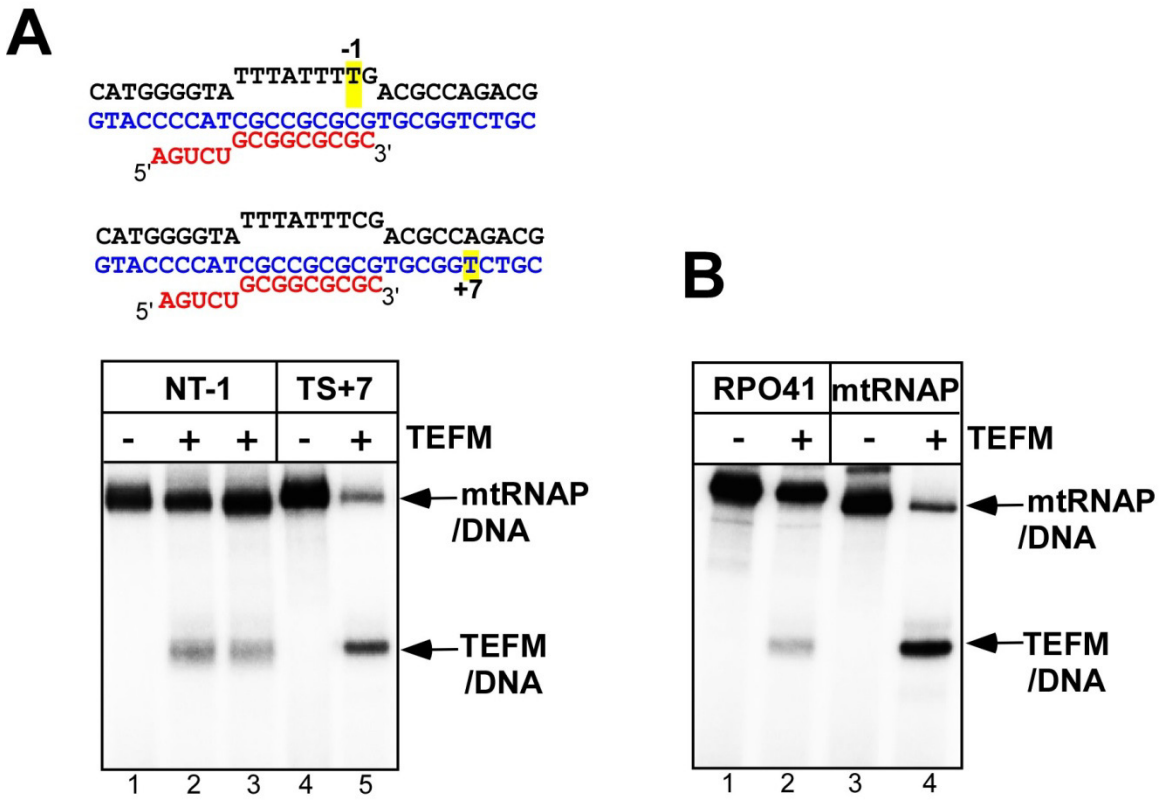


Figure S4. TEFM interacts with the downstream DNA in mtRNAP EC. (**A**) TEFM interacts with the downstream DNA. The ECs were assembled using scaffold containing 4-thio dTMP at position +7 (template strand, lanes 4-5) or -1 (non-template strand, lanes 1-3) of DNA. (**B**) Specificity of mtRNAP-TEFM interactions. ECs were assembled using either yeast mtRNAP (RPO41, lanes 1-2) or human mtRNAP (lanes 3-4) using scaffold containing 4-thio dTMP at position +7 in the template strand. Positions of the photo-reactive analog, 4-thio dTMP, is highlighted in yellow.

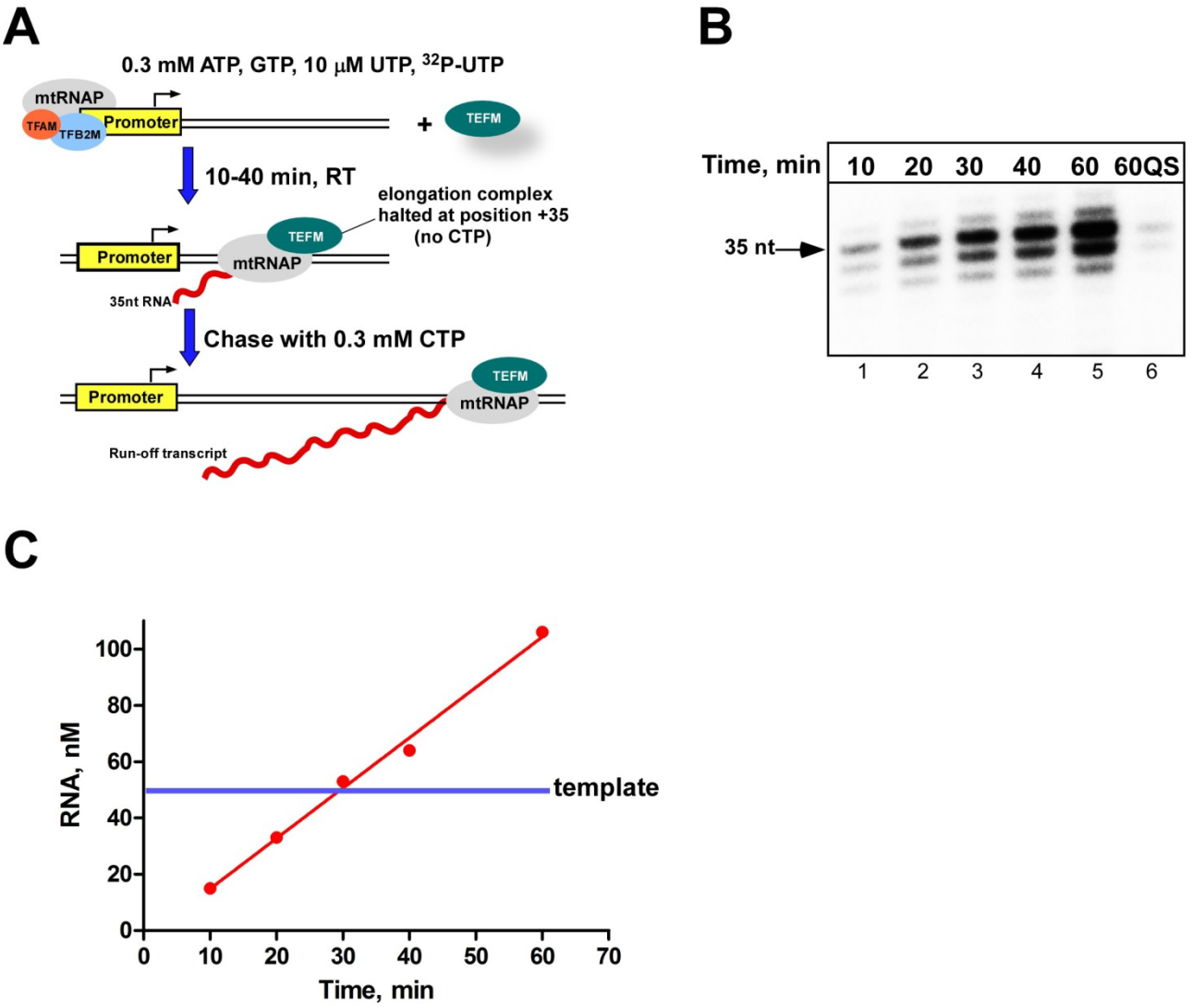


Figure S5. Halted MtRNAP ECs are unstable. A. Schematics of a halted complex stability experiment. The 35-mer start-up complexes were formed using the LSP promoter template (50 nM) containing no SCBII sequence by providing ATP (0.3 mM), GTP (0.3 mM), and UTP (0.1 mM) in the presence or absence of TEFM. **B**. Halted EC are unstable and dissociate. The halted complexes obtained as described above were incubated for time indicated (lanes 1-5) and resolved in 20% PAGE containing 6M urea. The EC incubated for 60 min was separated from the released RNA using QuickSpin G-50 column (lane 6). **C**. Accumulation of 35 nt RNA during transcription experiment above (B) indicates that the halted ECs are unstable.