Supplemental Material

Expression and purification of human mitoRNAP, TFA and TFB2

MitoRNAP and TFB2 were expressed using BL21-CodonPlus (DE3)-RIPL competent cells (Stratagene). TFA was 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 0.6 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. MitoRNAP was first purified by affinity chromatography on Ni-agarose beads (Qiagen) followed by heparin-sepharose purification in 250-1500 mM gradient of NaCl. TFA and TFB2 were purified using chitin-agarose (NEB) followed by heparin-sepharose purification in 250-1500 mM gradient of NaCl. The proteins were concentrated to 3-10 μM concentration, diluted 2 times with glycerol, aliquoted and stored at -70^0C.

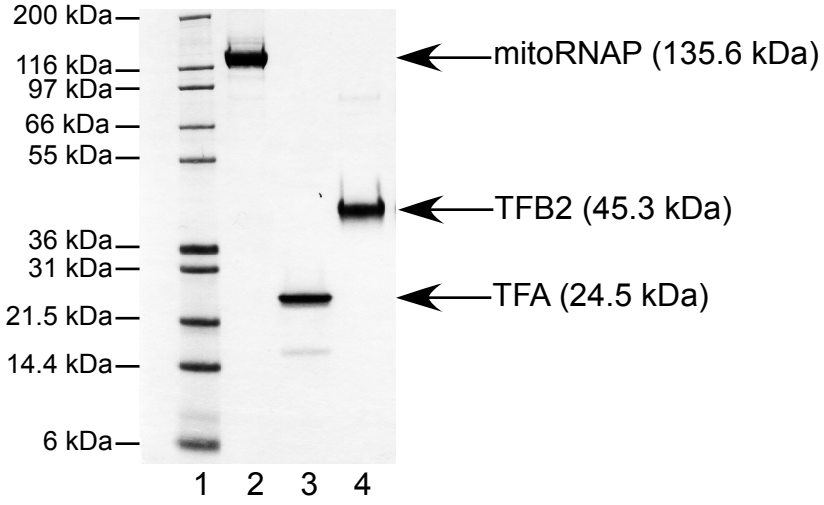

Figure S0. Purified recombinant components of human mitochondrial transcription machinery. Mark12 protein standards (Invitrogen, lane 1), human mitoRNAP (2 μg, lane 2), TFA (2 μg, lane 3) and WT TFB2 (2 μg, lane 4) were separated using 4-12% Bis-Tris SDS/MES gel (Invitrogen) and visualized by Coomassie staining.

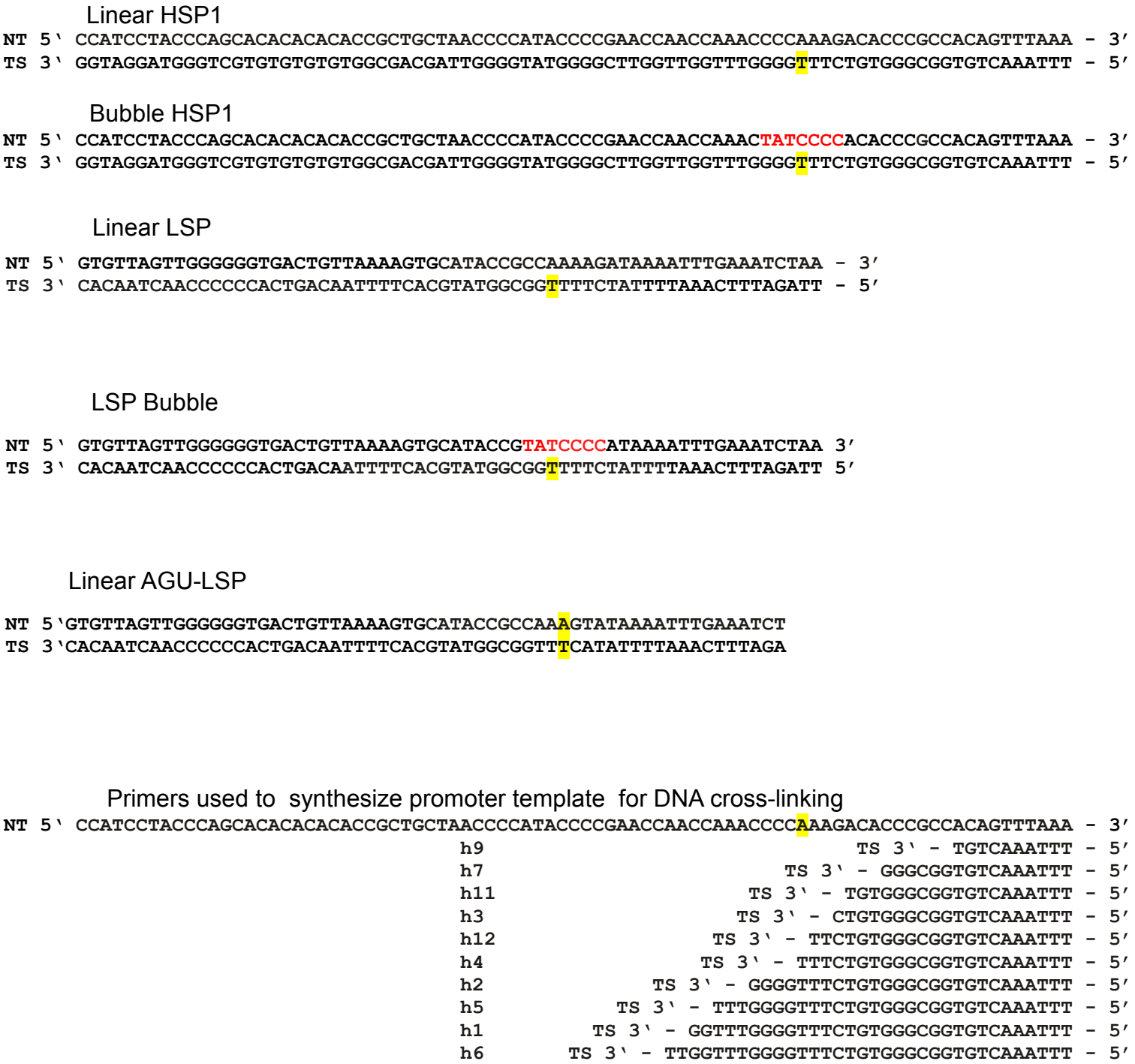

Figure S1. Templates used in transcription and crosslinking experiments

Sequence of the synthetic DNA templates containing the LSP and the HSP1 promoters is presented. Promoter start sites are indicated by yellow shades. Non-complementary region in promoter bubble templates is indicated in red. The primers used to synthesize promoter templates for DNA crosslinking are aligned against “templating” NT strand of the HSP1 promoter.

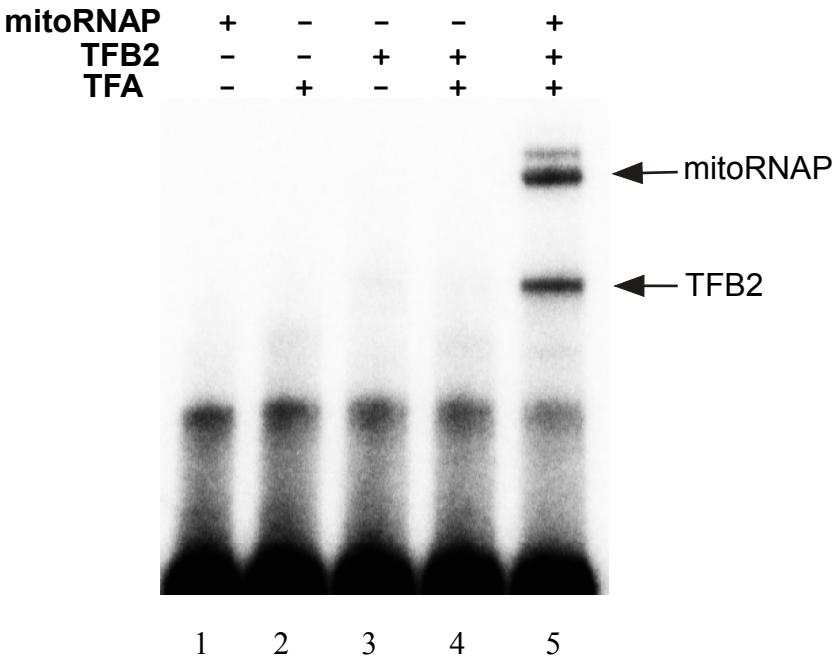

Figure S2. Protein-DNA crosslinking is specific to the presence of all components of the transcription initiation complex (IC)

The crosslinking was performed using a ^{32}P-labeled HSP1 template containing 4-thio dTMP incorporated at positions +1 to +3 downstream of the promoter start site and the proteins indicated. The products of the reaction were resolved using 4-12% Bis-Tris SDS/MES gel (Invitrogen).

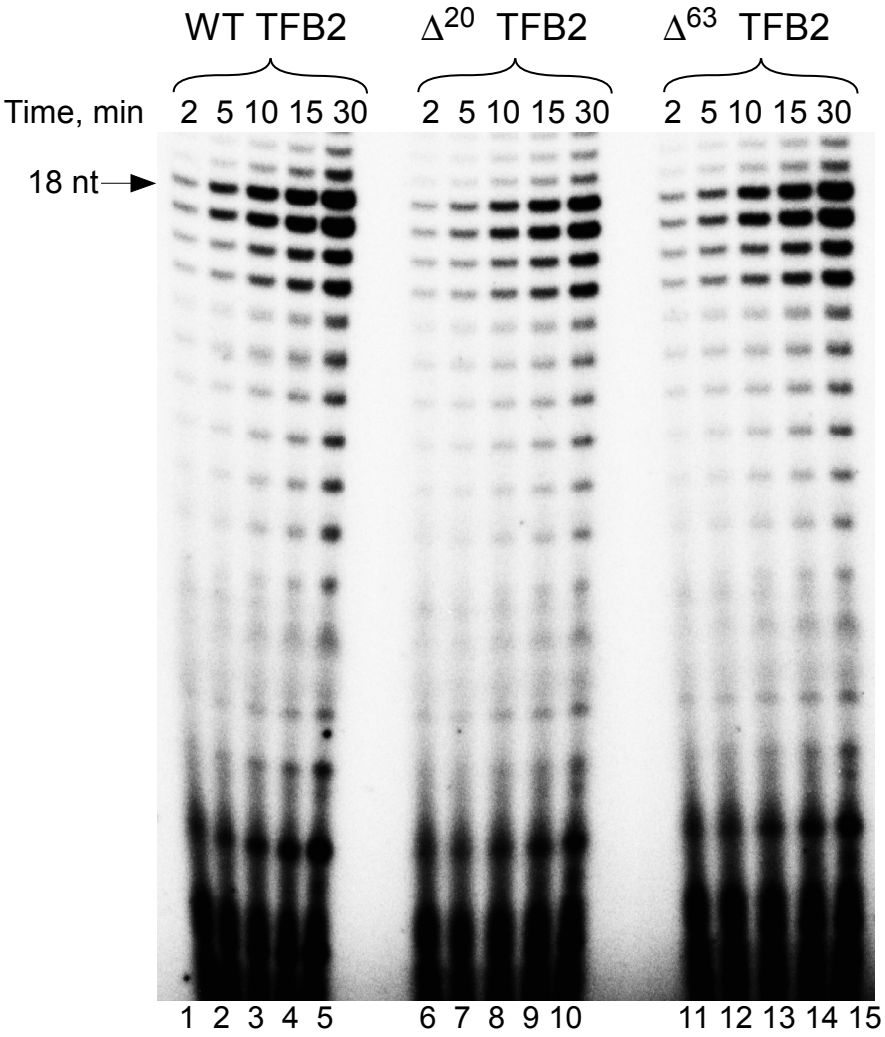

Figure S3. TFB2 deletion mutants exhibit the WT phenotype in run-off transcription assays

Run-off transcription assay using WT (lanes 1-5), Δ^{20} (lanes 6-10) and Δ^{63} (lanes 11-15) TFB2 was carried out as described in Materials and Methods using the LSP promoter template, ATP, [α-^{32}P] ATP, GTP and UTP. Transcription products were resolved using 20% PAGE containing 6M urea.

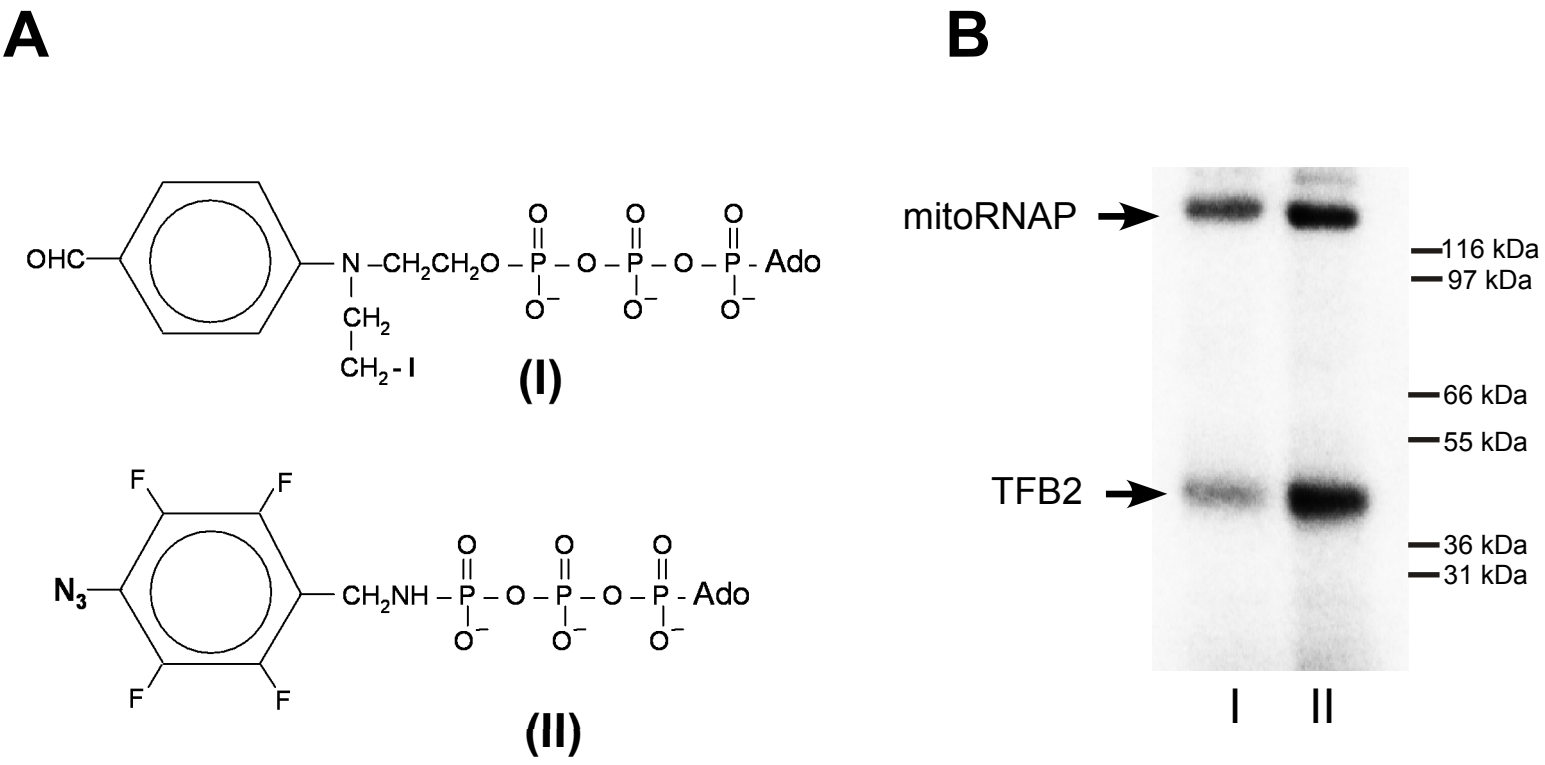

Figure S4. TFB2 crosslinks to a number of modified substrate analogs.

A. *Structural formulas of ATP derivatives used in auto catalytic experiments .*
Two ATP derivatives were used. The alkylating ATP derivative (compound I) was described previously [Severinov et al, 1997]. To obtain photo reactive 2,3,5,6-tetrafluoro-4-azido-benzylamido-ATP (compound II) the solution of ATP triethylammonium salt (8.2 mg, 10 mol) in 100 ml of DMF was treated with 10.4 mg (50 mol) of dicyclohexylcarbodiimide for 4 h and supplemented with 4.4 mg (20 mol) of 2,3,5,6-tetrafluoro-4-azido-benzylamine. The product was purified by TLC on silicagel plate in acetonitrile-water (4/1) developing system. The product was identified using UV spectroscopy and acidic hydrolysis (pH 2, 37^0C, 1 h), that yielded ATP and 2,3,5,6-tetrafluoro-4-azido- -benzylamine due to hydrolysis of phosphoramide bond.

B. Catalytic autolabeling of human mitochondrial IC
Catalytic autolabeling of the IC was performed as described in Material and Methods using 0.5 mM alkylating ATP derivative (lane 1) and 0.5 mM 2,3,5,6-tetrafluoro-4-azido-benzylamido-ATP (lane 2) in transcription buffer containing no magnesium ions for 30 min at RT. Crosslinking was initiated by addition of BH_4^- (1 mM, 10 min at RT) or by UV light irradiation (312 nM, 2 min at RT). The reactions were then immobilized on Ni-agarose beads and washed 6 times with the transcription buffer containing no magnesium ions. Labeling was achieved by addition of 1 μl of [α^{32}P]ATP (800 Ci/mM) and 10 mM $MgCl_2$ followed by incubation for 40 min at 35^0C. The products of the reaction were resolved using 4-12% Bis-Tris SDS/MES gel (Invitrogen).

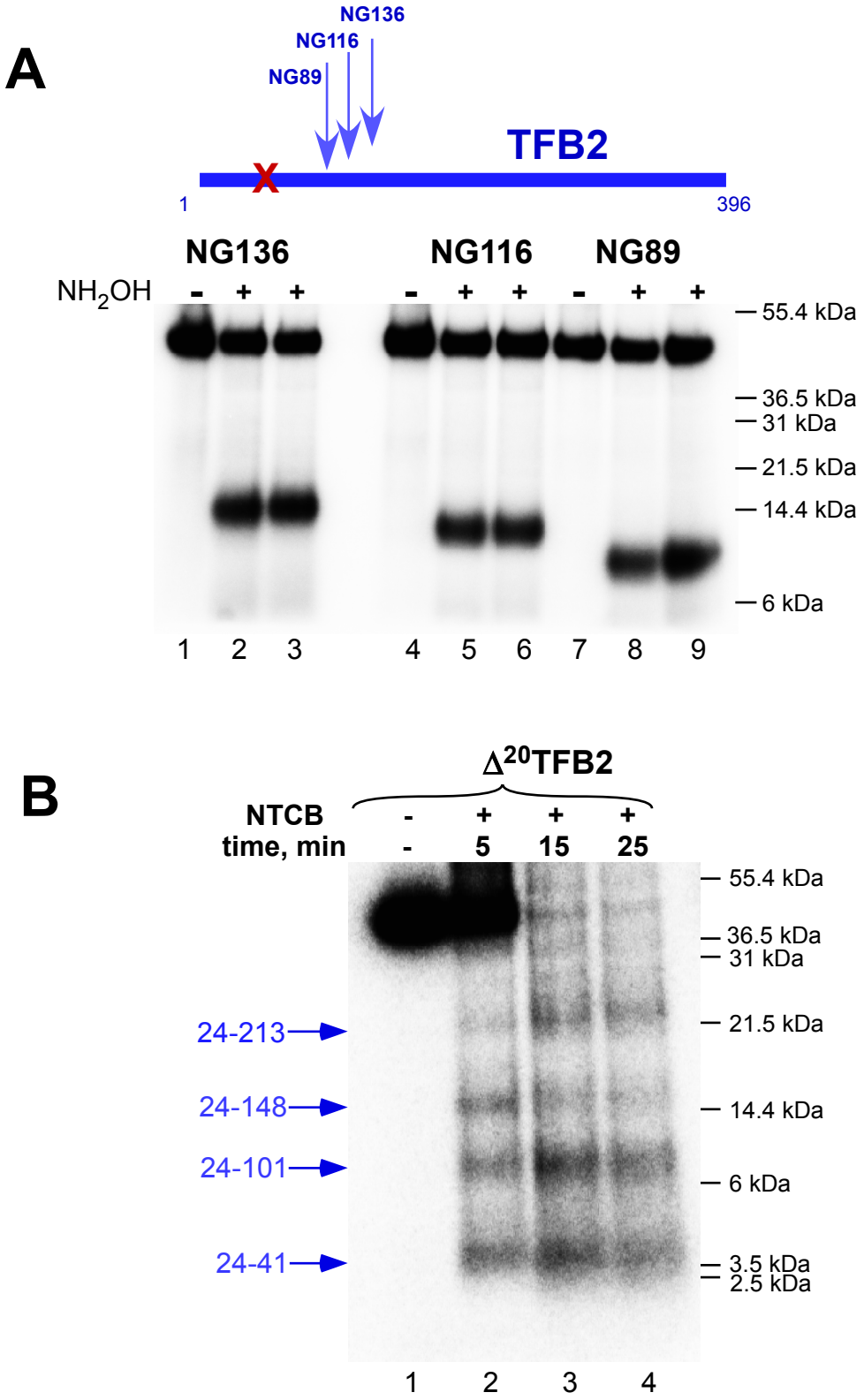

Figure S5. Mapping of the crosslink between TFB2 and the priming nucleotide.

A. Mapping of 2-hydroxybenzaldehyde AMP crosslink with hydroxylamine. Crosslinking of NG139, NG116 and NG89 TFB2 mutants with the AMP derivative was performed as described in Material and Methods. After the crosslinking, the reactions were treated with 0.2M hydroxylamine for 5 h (lanes 2,5,8) or 6 h (lanes 3,6,9) at 43^0C and resolved in 4-12% Bis-Tris SDS/MES gel (Invitrogen).

B. Mapping of 2-hydroxybenzaldehyde AMP crosslink with NTCB. Mutant Δ^{20}TFB2 was treated with NTCB for the time indicated at 37^0C and the products of the reaction were resolved using SDS PAGE as described above.

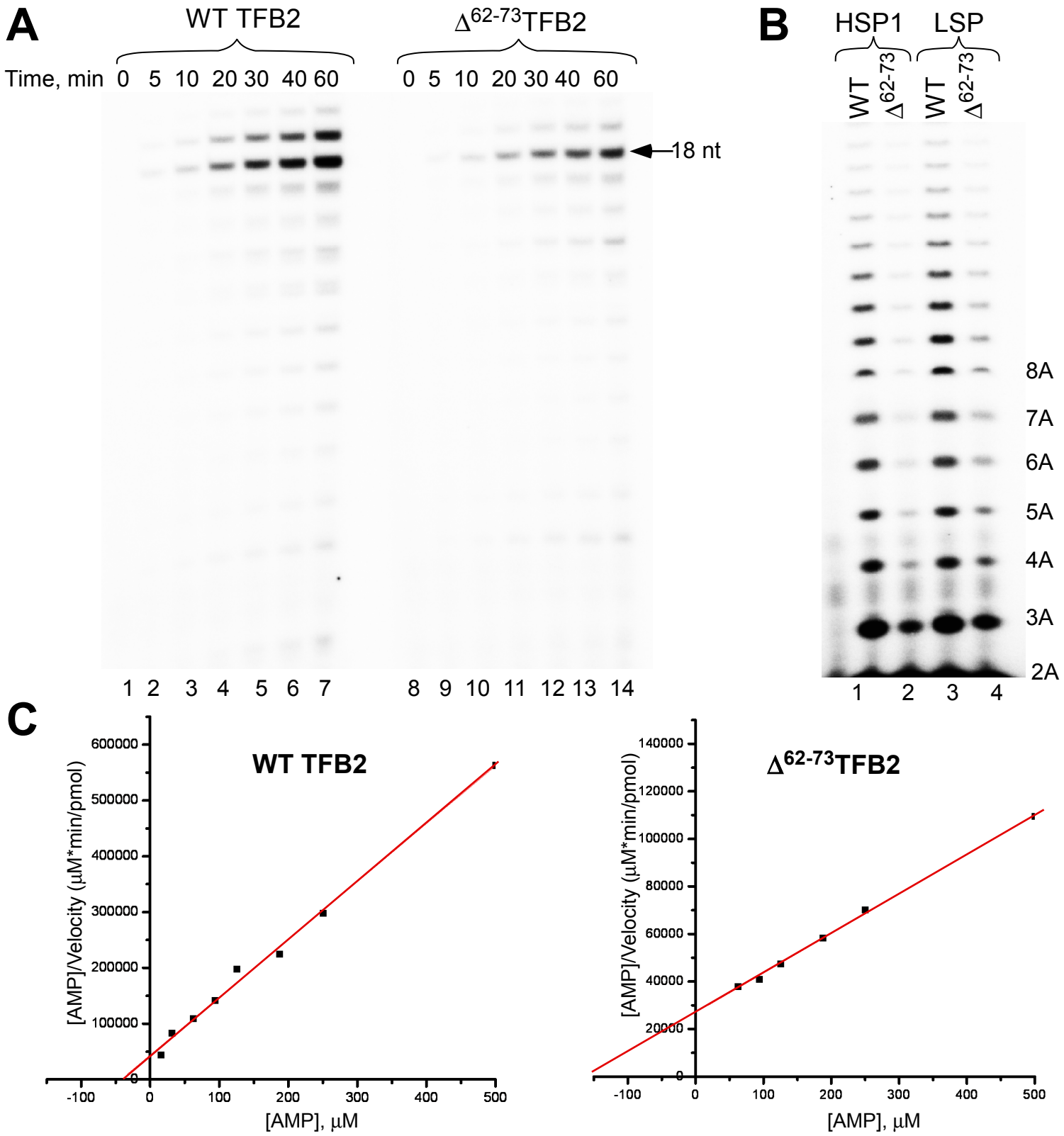

Figure S6. Properties of Δ^{62-73} TFB2 mutant. A. Run-off transcription assay using WT (lanes 1-7) or Δ^{62-73} (lanes 8-14) TFB2. Transcription was carried out as described in Materials and Methods using the LSP promoter template, ApApA primer, ATP, GTP and CTP. **B.** A-ladder production on LSP and HSP1 promoters by WT (lanes 1,3) or Δ^{62-73} (lanes 2,4) TFB2. **C.** Steady state kinetic experiments. Transcription reactions were performed using the LSP promoter in the presence of 1-1000 μM AMP (priming substrate) and 50 μM GTP to produce 5-mer transcripts. The data are presented using Hanes-Woolf plots for WT (left panel) and Δ^{62-73} (right panel) TFB2. K_m^{app} was calculated using the data obtained in three independent experiments.

References.

1. The sigma subunit conserved region 3 is part of “5‘-face” of active center of Escherichia coli RNA polymerase. Severinov K, Fenyö D, Severinova E, Mustaev A, Chait BT, Goldfarb A, Darst SA. J Biol Chem. 1994 Aug 19;269(33):20826-8.