Supplemental Material

A Model for Transcription Initiation in Human Mitochondria

Yaroslav I. Morozov[1], Andrey V. Parshin[1], Karen Agaronyan[1], Alan C.M. Cheung[2], Michael Anikin[1], Patrick Cramer[3] and Dmitry Temiakov[1*]

[1]*Department of Cell Biology, Rowan University, School of Osteopathic Medicine, 2 Medical Center Dr, Stratford, NJ 08084,USA*

[2]*Institute of Structural and Molecular Biology, University College London, Gower Street, London WC1E 6BT UK*

[3]*Max Planck Institute for Biophysical Chemistry, Department of Molecular Biology, Am Fassberg 11, 37077 Gottingen, Germany*

*Corresponding author: Dmitry Temiakov, email: d.temiakov@rowan.edu, tel: 856-566-6327

Includes:

Extended Materials and Methods,

Figures S1-S5,

Table S1

Extended Materials and Methods: **Synthetic promoter templates used in the course of the work**

LSP promoter

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                                                                  +1
GAGTGGGAGGGGAAAATAATGTGTTAGTTGGGGGGTGACTGTTAAAAGTGCATACCGCCAAAAGATAAAATTTGTGGGCC  hLSP-60
```

HSP1 promoter

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                                                                +1
CCATCCTACCCAGCACACACACACCGCTGCTAACCCCATACCCCGAACCAACCAAACCCCAAAGACACCCGCCACAGTTTAAA h60NT1 WT
```

templates for DNA cross-linking

LSP-template

```
                                                                +1
GCGGCGATCTCGATTCAGACTGTTAGTTAGGGAGTGACTGTTAAAAGTGCATACCGCCAAAAGATAAGG
CGCCGCTAUAGCTAAGTCTGACAATCAATCCCTCACTGACAATTTTCACGTATGGCGGTTTTCTATTCC
```

TS35sU

non-promoter template

```
GCGGCGATCTCGATTCAGACCGCCCTCTGGTTCTTGGCGGATCCTCCAATGGGGAAGGACACGGACGACC
CGCCGCTAUAGCTAAGTCTGGCGGGAGACCAAGAACCGCCTAGGAGGTTACCCCTTCCTGTGCCTGCTGG
```

TS35sU

+1 thioU LSP template

```
                                                      +1
5'-GTGTTAGTTGGGGGGTGACTGTTAAAAGTGCATACCGCCAAAAGATAAAATTTGTGGGCC-3'
3'-CACAATCAACCCCCCACTGACAATTTTCACGTATGGCGGTUTTCTATTTTAAACACCCGG-5'
```

-5 thioG LSP template

```
                                                      +1
5'-GTGTTAGTTGGGGGGTGACTGTTAAAAGTGCATACCGCCAAAAGATAAAATTTGTGGGCC-3'
3'-CACAATCAACCCCCCACTGACAATTTTCACGTATGGCGGTTTTCTATTTTAAACACCCGG-5'
```

-9 thioU LSP template

```
                                                      +1
5'-GTGTTAGTTGGGGGGTGACTGTTAAAAGTGCATACCGCCAAAAGATAAAATTTGTGGGCC-3'
3'-CACAATCAACCCCCCACTGACAATTTTCACGUATGGCGGTTTTCTATTTTAAACACCCGG-5'
```

-10 thioG LSP template

```
                                                      +1
5'-GTGTTAGTTGGGGGGTGACTGTTAAAAGTGCATACCGCCAAAAGATAAAATTTGTGGGCC-3'
3'-CACAATCAACCCCCCACTGACAATTTTCACGTATGGCGGTTTTCTATTTTAAACACCCGG-5'
```

U = 4-thio UMP **G** = 6-thio dGMP

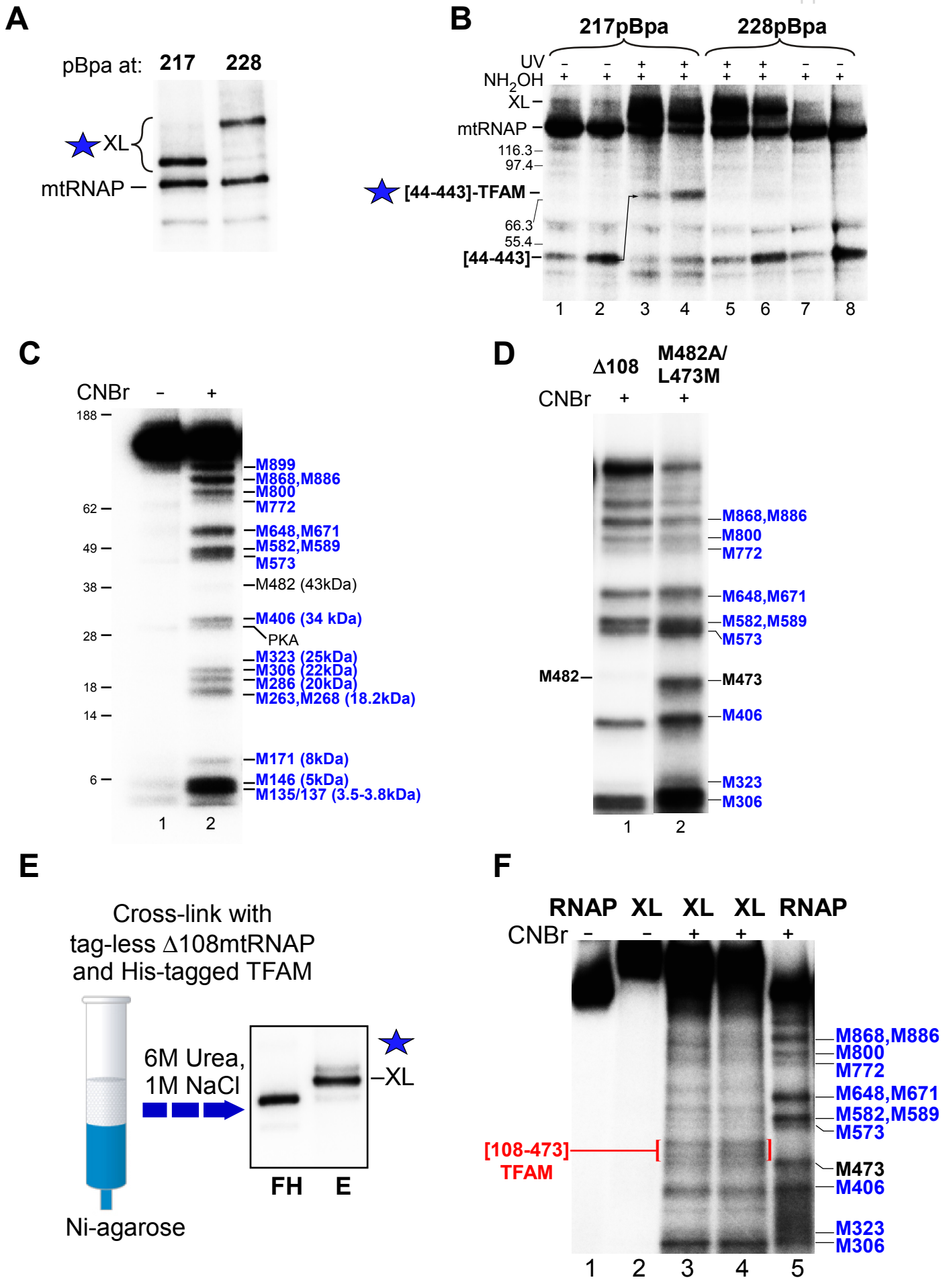

Figure S1. Mapping of TFAM-228pBpa cross-links to mtRNAP (related to Figure 1)

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A. Cross-links at 217 and 228 have different mobility in SDS-PAGE. The pre-ICs (50 nM) were assembled with ^{32}P-labeled mtRNAP, LSP and 217pBpaTFAM or 228pBpaTFAM and UV irradiated for 30 min. The products of the reaction were resolved using 6% Tris-glycine SDS PAGE.

B. Mapping of the 228pBpa-TFAM cross-link using hydroxylamine. The pre-ICs were assembled using ^{32}P-labeled mutant mtRNAP having a single NG pair at position 443 and 217pBpa or 228pBpa-TFAM. The complexes were treated with hydroxylamine for 2-4 h at 43^{0}C prior (lanes 1,2, 7 and 8) or after (lanes 3-6) UV-irradiation, and the products of the reaction resolved using 4-12% Bis-Tris MES-SDS gel.

Explanation of the cross-link mapping data. *In reaction that serves as a control, treatment of 217pBpaTFAM - ^{32}P-labeled NG443 mtRNAP cross-link with hydroxylamine results in a shift of the band representing the N-terminal mtRNAP fragment (residues 44-443, lane 1 and 2 in panel B) by a TFAM mass (lanes 3,4). This is consistent with our previous finding that the 217MPB-modified TFAM cross-link is to the N-terminal extension region of mtRNAP (13). In contrast, cleavage of 228pBpaTFAM-mtRNAP/DNA cross-link did not result in any noticeable shift of the 43-443 band (compare lanes 6 and 8, panel B), suggesting that this cross-link is to region 444-1230 of mtRNAP.*

C. Fine mapping of the 228pBpa-TFAM cross-linking using CNBr.

CNBr cleavage of ^{33}P-labeled Δ119 mtRNAP. The labeled mtRNAP was treated with CNBr as described in Experimental Procedures. The products of the reaction were resolved using 4-12% Bis-Tris MES-SDS gel (Invitrogen). Positions of SeeBlue protein standards (Invitrogen) are indicated to left. The bands were assigned based on their relative mobility in PAGE and theoretical molecular weight. Note that the cleavage at M482 residue is about 10 times less efficient than at the other methionines. Auto labeling of PKA enzyme (NEB) results in appearance of a low intensity band in both control and experimental lanes.

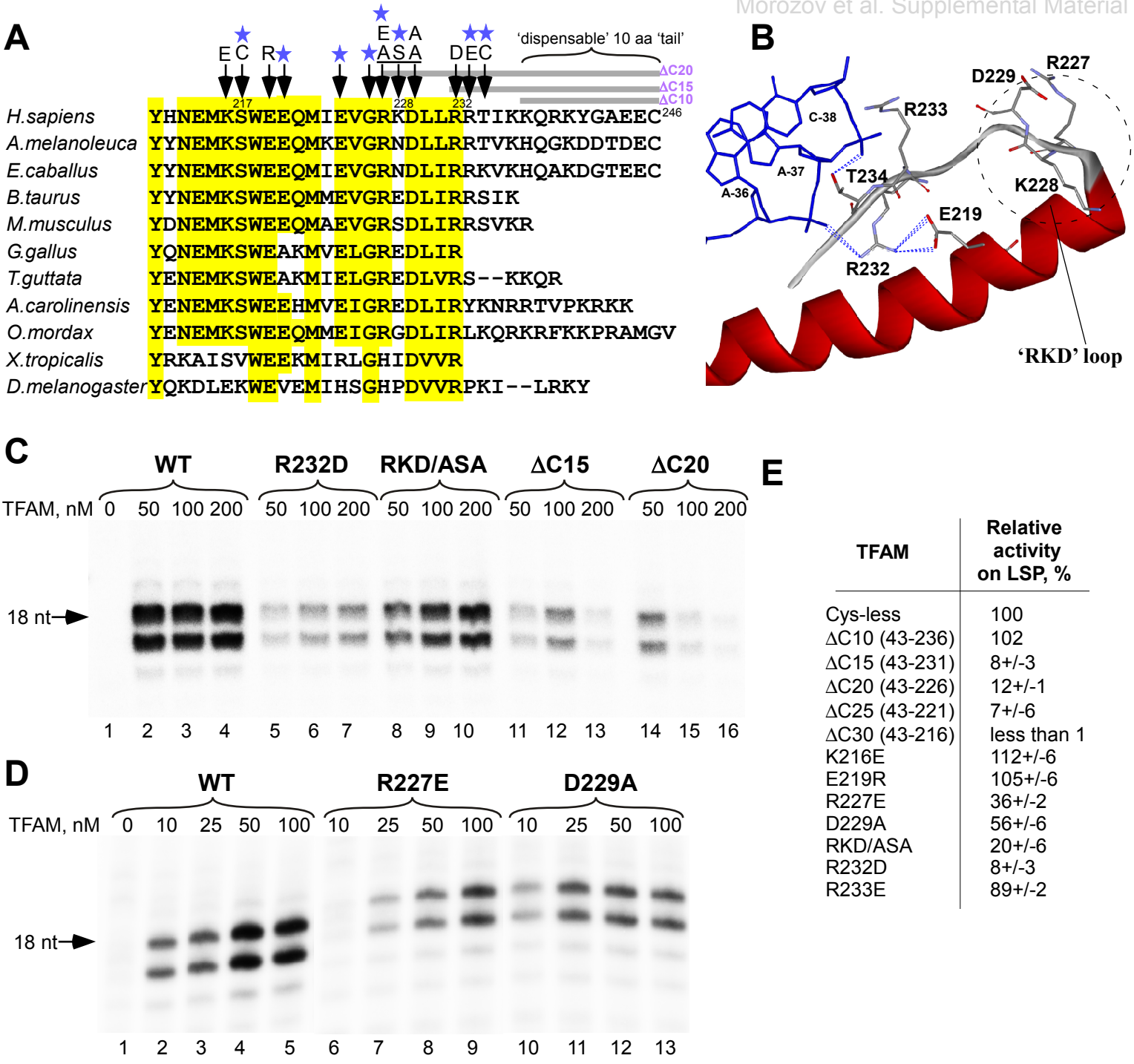
Figure S1 (continued). Mapping of TFAM-228pBpa cross-links to mtRNAP (related to Figure 1)

D. CNBr cleavage pattern of M482A/L473M mutant mtRNAP. To avoid ambiguity in CNBr pattern interpretation, the poorly cleavable M482 residue was substituted by alanine and a new methionine at position 473 was introduced in Δ108 (tag-less) mtRNAP. The cleavage pattern of ^{32}P-labeled M482A/L473M mutant reveals a new band, corresponding to 109-473 peptide clearly visible on the PAGE autoradiogram.

E. Purification of cross-linked species on Ni-agarose column. The pre-IC were formed using tag-less ^{32}P-labeled Δ108 mtRNAP (res 109-1230) in which an additional methionine residue (M473) was introduced and his-tagged pBpa228-TFAM. After UV-irradiation, the cross-linking reaction was passed through Ni-agarose column, washed 6 times with denaturing solution (6M urea, 1M NaCl) and the cross-link eluted with 0.2 mM imidazole (lane 'E').

F. Mapping of the pBpa228-TFAM-mtRNAP cross-link. The isolated cross-linked material (lane 2) was treated with CNBr for 5 (lane 3) or 10 (lane 4) min and loaded onto 4-12% Bis-Tris MES-SDS gel along with CNBr-treated ^{32}P-labeled mtRNAP (lane 5).

Explanation of the cross-link mapping data. *The cleavage pattern of the isolated cross-link (lanes 3,4) was compared to the cleavage pattern of ^{32}P-mtRNAP (lane 5; see also panel B for band assignment). The key difference in these patterns is at M473 (indicated by red brackets and numbers), as there are two additional cross-linked species clearly visible above the band that represents peptide 109-473 (compare lanes 3,4 and lane 5), suggesting that the mobility is changed due to cross-linking of TFAM molecules to the 406-473 interval of mtRNAP. These data, together with hydroxylamine cleavage data (cross-link region is to the region 444-1230,* ***Fig. S2****), indicate that the 228pBpaTFAM cross-linking site is located in the region 444-473 of the D-helix in the promoter-binding domain of mtRNAP.*



E

TFAM	Relative activity on LSP, %
Cys-less	100
ΔC10 (43-236)	102
ΔC15 (43-231)	8+/-3
ΔC20 (43-226)	12+/-1
ΔC25 (43-221)	7+/-6
ΔC30 (43-216)	less than 1
K216E	112+/-6
E219R	105+/-6
R227E	36+/-2
D229A	56+/-6
RKD/ASA	20+/-6
R232D	8+/-3
R233E	89+/-2

Figure S2. C-terminus of TFAM is involved in interactions with mtRNAP (related to Figure 1)

A. Sequence conservation in the mtRNAP-binding region of TFAM of different species. Black arrows and letters indicate point mutations made; black arrows and stars indicate pBpa substitutions. Blue lines at the top of the panel illustrate the extent of C-terminal deletions in TFAM.

B. Location of a conserved RKD loop in the C-terminal domain of TFAM (PDB ID 3TMM).

C, D. Mutations in the C-terminus of TFAM affect its activity. In vitro transcription assay was performed as described in Material and Methods using the LSP promoter template.

E. Summary of transcription activity of various TFAM mutants.

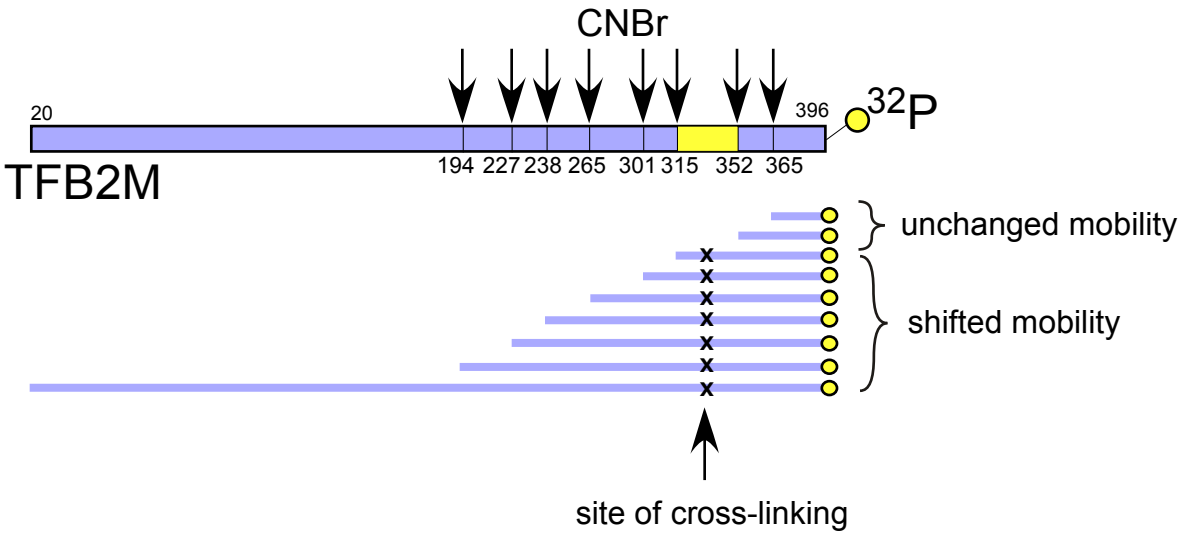


Figure S3. Analysis of TFB2M-mtRNAP cross-linking mapping data (related to Figure 2A). Cross-linking mapping with CNBr (cleaves after Met residues) was performed under "single-hit" conditions (19, 20), i.e. when each TFB2M molecule was cleaved only once, on average. Cleavage of a C-terminally ^{32}P-labeled TFB2M generated a characteristic C-terminal pattern of the radiolabeled products (Figure 2A, lanes 4,5). Note a similar intensity of the cleavage products of TFB2M generated by CNBr.

When TFB2M-mtRNAP cross-link is cleaved, the cross-linked products have much larger molecular weights due to addition of the mtRNAP peptides (mtRNAP is also cleaved and generates unlabeled products) and thus appear shifted on the SDS gel (lanes 2,3 in Figure 2A). The mobility of non-cross-linked peptides remains unchanged. *The cross-linking site is defined as the interval between the N-terminal residue of the largest peptide with unchanged mobility and the N-terminal residue of the smallest shifted cleavage product.* Therefore, in the experiment shown in Figure 2 the site of cross-linking is between residues 316 and 352. Note that some de-cross-linking (<10%) occurs during isolation of the TFB2M-mtRNAP cross-link (Figure 2A, lane 1).

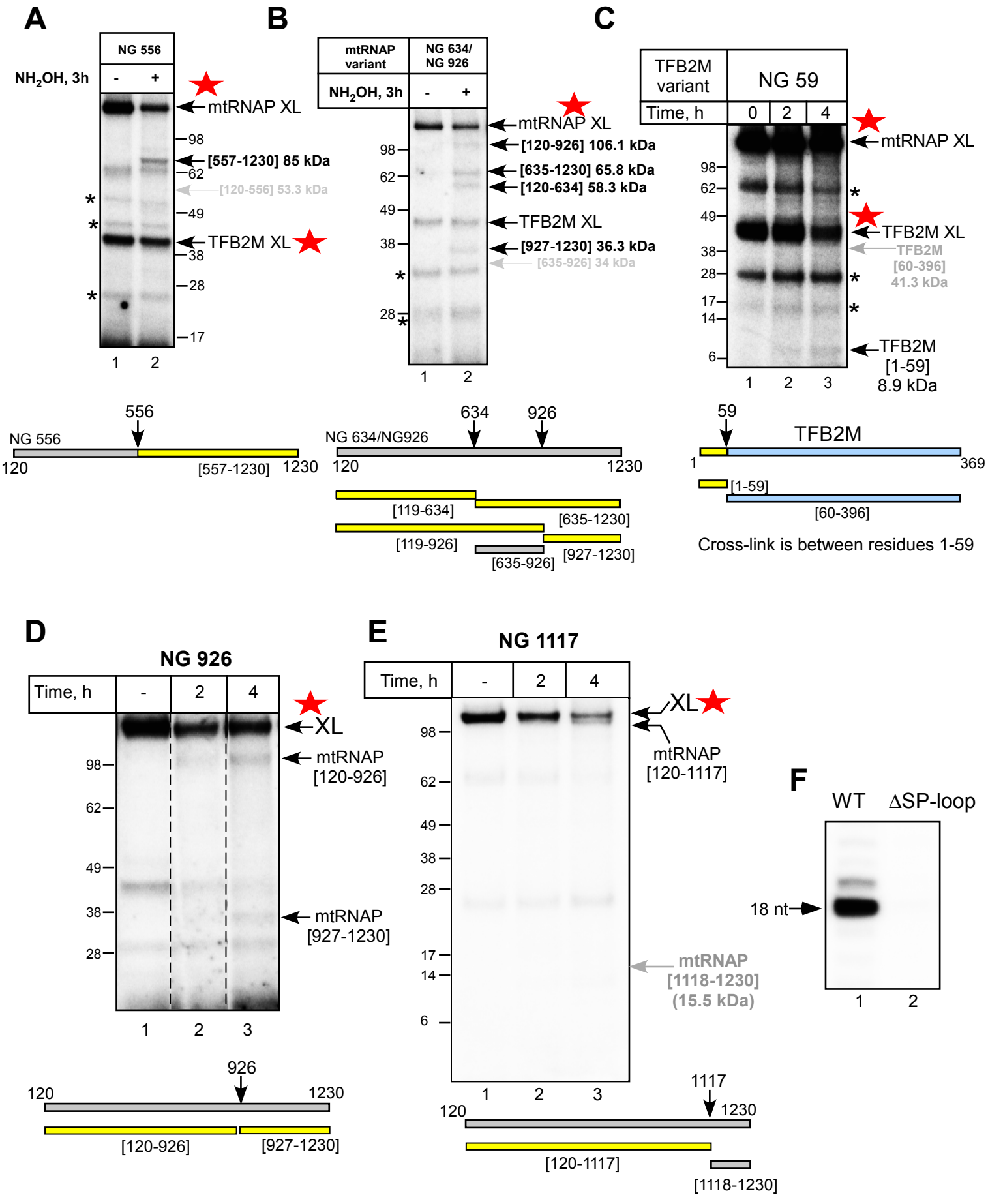


Figure S4. Mapping of mtRNAP interaction with the promoter (related to Figure 2,3).

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A. Mapping of NG556 mtRNAP cross-link at base -5 using hydroxylamine cleavage. Initiation complexes (250 nM) containing ^{32}P-labeled template with 6-thio dGMP at position -5 were UV-irradiated for 15 min. Upper panel. The cross-linking was performed using Δ119 mtRNAP having NG pair at position 556. The grey arrow marks the expected position of 120-556 fragment of mtRNAP. The asterisks indicated non-specific cross-linking bands present in all lanes. Mol. weight of protein markers (SeeBlue, Invitrogen) is indicated to the right of the panel (kDa). Lower panel. The scheme illustrates possible products of hydroxylamine cleavage; yellow boxes indicate products appeared labeled on the gel.

B. Mapping of NG634/926 mtRNAP cross-link at base -5 using hydroxylamine cleavage. Complex formation and cross-linking was performed as in panel A using Δ119 mtRNAP having NG pairs at positions 634 and 926. The grey arrow marks the expected position of 635-926 fragment of mtRNAP.

Explanation of the cross-link mapping data. *Cleavage of the NG556 mtRNAP generate a single labeled fragment corresponding to the C-terminal region of mtRNAP (panel A, lane 2). Cleavage of NG634/926 mtRNAP generates several labeled peptides; however the N-terminal labeled peptide corresponds to the region 120-634 of mtRNAP. Taken together, these data suggest that the cross-link from base -5 is to the interval 557-634 of mtRNAP. This region includes the intercalating hairpin of mtRNAP, the structural element in single-subunit RNAPs known to interact with this position in promoter DNA (17). The presence of the very C-terminal labeled peptide (927-1230, Figure S4B) indicates a second cross-linking site with the -5 DNA base. From this region of mtRNAP, which is located ~30-50 A away from -5 DNA base, only one structural element (the specificity loop, res 1086-1110) can possibly reach promoter DNA (17).*

C. Mapping of DNA-TFB2M cross-link at base -5 using hydroxylamine cleavage. Complex formation and cross-linking was performed NG-less Δ119 mtRNAP variant. Lower panel. The scheme illustrates possible products of hydroxylamine cleavage; yellow boxes indicate products that appeared labeled on the gel. The asterisks indicated non-specific cross-linking bands present in all lanes. Mol. weight of the protein markers (SeeBlue, Invitrogen) is indicated to the left of the panel (kDa).

Explanation of the cross-link mapping data. *Cleavage of the NG59 TFB2M mutant generates a single labeled fragment that corresponds to the N-terminal region of TFB2M (lanes 2-3, note accumulation of the product), suggesting that DNA-TFB2M cross-link site is located between residues 1-59 (B, lanes 2 and 3).*

Figure S4 (continued). Mapping of mtRNAP interaction with the promoter (related to Figure 2,3).

D. Mapping of mtRNAP (NG926) interactions with the -10 template base in the IC. Initiation complexes (250 nM) containing 6-thio dGMP at position -10 in the template DNA strand were UV-irradiated for 15 min. Upper panel. The cross-linking was performed using Δ119 mtRNAP having NG pair at position 926 and the products resolved in 4-12% SDS PAGE. Mol. weight of the protein markers (SeeBlue, Invitrogen) is indicated to the left of the panel (kDa). Lower panel. Scheme illustrating possible products of hydroxylamine cleavage; yellow boxes indicate specific products appeared labeled on the gel.

E. Mapping of mtRNAP (NG1117) interactions with the -10 template base in the IC. Complex formation and cross-linking was performed as in panel A but using Δ119 mtRNAP having NG pair at position 1117. Lower panel. Scheme illustrating possible products of hydroxylamine cleavage, yellow boxes indicate products appeared labeled on the gel.

Explanation of the cross-link mapping data. *Cleavage of the NG926 mtRNAP mutant generates two labeled fragments corresponding to the N- and C-terminal regions of mtRNAP (panel A), while cleavage at NG1117 (panel B) produces a band corresponding to the N-terminal fragment only (residues 120-1117). Taken together, these results suggest that there are two DNA cross-linking sites in mtRNAP : between residues 120-926 and 927-1117 (A, B). The latter site includes the specificity loop of mtRNAP, the only structural element in the C-terminus of mtRNAP that can reach the -10 DNA base (17).*

F. Specificity loop of mtRNAP is important for transcription initiation.

Transcription assay was performed using the LSP promoter template (50 nM), 150 nM WT (lane 1) or specificity loop deletion mutant (ΔSP, lane 2) of mtRNAP, 150 nM TFB2M and 50 nM TFAM in the presence of ^{32}P-labeled pApApA primer, ATP (0.01 mM), GTP (0.3 mM) and UTP (0.05 mM) for 30 min at 35^{0}C.

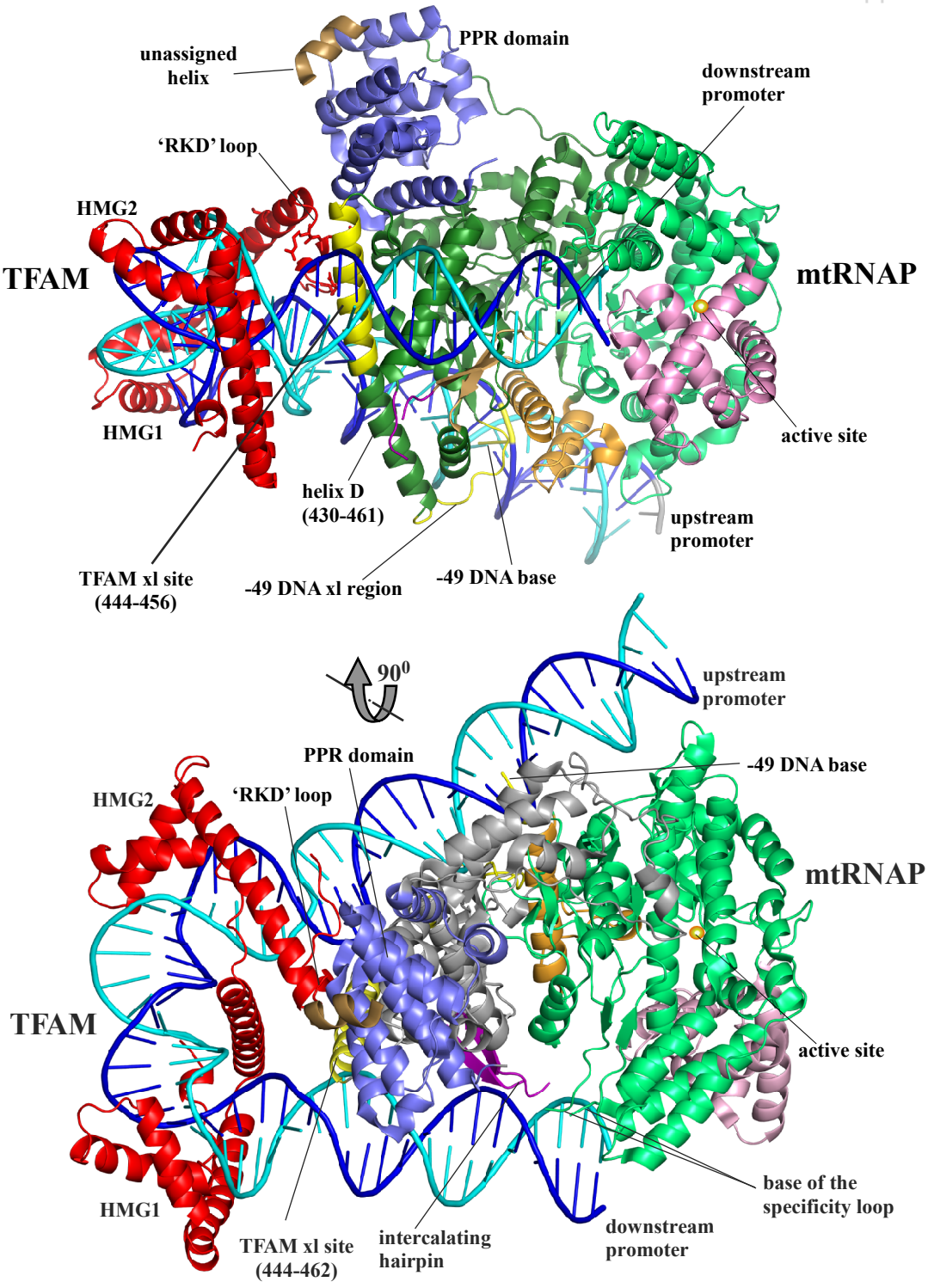

Figure S5. Model of the pre-initiation complex (related to Figure 4). Two experimental data sets (mtRNAP, PDB ID 3SPA and DNA/TFAM complex, PDB ID 3TMM) were used to model the pre-IC based on biochemical data. The upstream and downstream promoter DNA regions (depicted as ribbons, T-strand, blue, NT-strand, cyan) were extended to emulate trajectory of the nucleic acid in the pre-IC. MtRNAP is depicted as a ribbon (NTD, dark green; palm, light green; fingers, pink; intercalating hairpin, purple; PPR domain, slate; unassigned helix, sand). A Mg^{2+} ion (orange) was placed according to a T7 RNAP structure. TFAM is shown in red (ribbon). The TFAM and -49 DNA cross-linking sites in mtRNAP are indicated in yellow.

Table S1. Activity of mtRNAP variants having substitution in the region of the D helix

mtRNAP[1]	Relative transcription activity on LSP[2], %
Δ119 (120-1230)	100
PKA Δ119	102+/-2
E447pBpa	ND[3]
R450pBpa	89+/-4
E451pBpa	ND
K453pBpa	ND
N454pBpa	92+/-3
Y461pBpa	105+/-2
Y468pBpa	91+/-2
E457K	95+/-4
E457A	101+/-3
Y461A	106+/-2
Y468A	93+/-2

[1] E447pBpa, E451Bpa,K453Bpa and E457K substitution mutants were made in PKA Δ119 mtRNAP background, all others mutants- in Δ119 mtRNAP.

[2] Activity of mtRNAP mutants was measured using transcription assay. The reactions contained 50 nM LSP template, 50 nM mtRNAP, 50 nm TFB2M, 50 nM of TFAM and nucleotide mixtures described in Experimental Procedures.

[3] ND- activity was not determined.