

Peer Review File

Structural Basis for Substrate Binding and Selection by Human Mitochondrial RNA Polymerase



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

To review this manuscript, I examined the text, two sets of coordinates and underlying density maps, 3 figures, 3 movies, 5 supplementary tables, 1 supplementary table, and 2 PDB validation reports.

RNA polymerases mediate the transfer of information from DNA to RNA. In contrast to the eukaryotic nuclear RNA polymerases, phage and mitochondrial enzymes feature an uncomplicated architecture. The phage and mitochondrial RNA polymerase are assembled from a single catalytic subunit; eukaryotic mitochondrial RNA polymerases also feature a transcription elongation factor. Prior structural analyses of phage and mitochondrial RNA polymerase have established strong links to the mechanisms of family A DNA polymerase (the phage and mitochondrial RNA polymerases are also members of family A). Nucleic acid polymerases exhibit a common architecture through their resemblance to a human right hand, with sub-domains termed palm, thumb, and fingers. Catalysis and quality control are mediated by the palm domain; the thumb binds the product duplex; the fingers domain binds the template and incoming nucleotide and mediates quality control. The fingers sub-domain undergoes an open-to-closed cycle during the catalytic cycle.

To better understand the transcriptional cycle implemented by the human MT RNA polymerase, Herbine et al used cryogenic electron microscopy to determine 2 structures of the MT RNA polymerase bound to a model transcription elongating substrate and the next incoming nucleotide.

Herbine et al come to the following major findings:

- 1) Two cryo-EM structures of the human mitochondrial RNA polymerase (MT-RNAP), the two-protein subunit transcription elongation factor (TEFM), an RNA-DNA hybrid with an unpaired 8 bp sequence (so-called transcription bubble), and the non-hydrolysable ATP analog (AMP-C-P). The resolutions of these structures are 2.77 and 2.59 Å. The cryo-EM analysis is of high quality.
- 2) Both structures (termed ONE and TWO), feature the MT-RNAP, the nucleic substrate, and the ATP analog. The two structures (protein and DNA) follow each other closely, except as discussed below. Both structures exhibit a flipped base on the non-template into a binding site formed out of residues from the TEFM. In both structures, the DNA-RNA sub-structure is found in the post-translocated step; ready for the next round of insertion.
- 3) The first structure (termed ONE) is the Substrate Entry Complex. The ATP analog is bound by the 'fingers' sub-domain. Relative to structure TWO, the ATP analog in structure ONE is in a distinct pre-insertion site. The position and configuration of the ATP analog is not compatible with catalysis/insertion. No magnesium ions are seen in the structure. The fingers are in the open configuration. The authors also see several interactions reminiscent of those seen with DNA polymerase dsDNA complexes.
- 4) The second structure (termed TWO) is the Substrate Insertion complex. The ATP analog is in the insertion site, stacked on the base most recently incorporated. The incoming ATP analog is also base

paired with the next template base to be copied. The fingers sub-domain has now closed on top of the nascent base pair. And, two magnesium ions appear in the active site. This complex is also reminiscent of conformers of DNA polymerase.

5) The structure-suggested roles of two residues (Q992 and Y999) are probed using biochemical studies.

Given that the determined structures resemble others from DNA polymerase systems, a more detailed comparison would provide greater context and validation of the present work.

Reviewer #2 (Remarks to the Author):

Mitochondria uses phage-type RNA polymerase (mtRNAP) for transcribing mtDNA. In this study, authors used human mtRNAP to investigate substrate NTP loading to the active site and associated conformational changes of mtRNAP, DNA template and NTP by determining two cryo-EM structures of mtRNAP elongation complex (EC) with incoming NTP, named substrate entry complex (PDB: 8U8U) and substrate insertion complex. What new in this structural work compared with previous works is capturing the substrate entry complex, which shows for the first time that NTP binds O-helix of finger domain but not form base pair with DNA template, suggesting a mechanism of substrate selection prior to base pairing with DNA template. Finding is very interesting and provide new insight into the substrate selection of mtRNAP and related enzymes.

However, unfortunately, qualities of cryo-EM density map of both complexes and the final models are not good enough to claim new findings. For examples, densities of finger domain and NTP are relatively poor and the final model including some important residues (Y999, M995, Y956 and NTP [no density covering sugar, extra density next to alpha phosphate] in 8U8U; no density connecting between phosphate and sugar of NTP, somehow RNA 3'-OH and alpha phosphate of NTP is connected, both MgA and MgB do not fit density in 8U8V). I think lack of high-quality density around finger and NTP is due to mobility of finger and also particles belonging to each state were not separated well during cryo-EM data processing. Authors have to find better solution to separate particles and improve cryo-EM maps. One way is soaking non-cognate NTP to EC, which may form only substrate entry complex due to lack of base pairing with template DNA. Alternatively, increase concentration of AMPCPP (from 1 to 10 mM) to convert all EC to substrate insertion complex.

One of distinct features of the substrate entry complex compare with other elongation complex structures determined before is that the template DNA base at i+1 position (T) is inserted between O- and Y-helices. If authors aim to claim this is a general mechanism, they have to determine the structures of elongation complex containing different DNA bases at i+1 position (A, G, or C). DNA base at i+1

position interacts with Q1009, Q992 and T996 residues, thus functions of these residues have to be validated by site-directed mutagenesis.

Reviewer #3 (Remarks to the Author):

In this manuscript, authors reported two cryoEM structures of human mitochondrial transcription elongation complexes that reveal substrate ATP bound in Entry and Insertion Sites, respectively. The entry site was not reported in any single-subunit RNAPs, in which the phosphate moiety is recognized by RNAP, the nucleobase moiety is not. This is different from previous reported pre-insertion state of T7 RNAP. In the insertion state, the substrate insertion state, the substrate base establishes Watson-Crick base pair the template base at the active site, the fingers domain is in closed state. Based on two new CryoEM structural data and previous reported pre-insertion state of T7 RNAP, authors propose a model for how human mitochondrial RNA polymerase recognize and incorporation substrate during transcription elongation. This work provides important mechanistic insights into human mitochondrial transcription. The manuscript is well-written. The manuscript will be of general interest to the transcription field and broad readership of Nature Communications. One minor comment is listed here.

Could authors comment what factors make the new entry state is more stable for human mitochondrial transcription elongation complex, therefore authors are able to captured in this study, whereas no such state is captured for T7 RNAP or other single-subunit RNAP?

REVIEWER COMMENTS

Authors: We thank all reviewers for the constructive criticism that helped us improve the manuscript.

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sub-domain. Relative to structure TWO, the ATP analog in structure ONE is in a distinct pre-insertion site. The position and configuration of the ATP analog is not compatible with catalysis/insertion. No magnesium ions are seen in the structure. The fingers are in the open configuration. The authors also see several interactions reminiscent of those seen with DNA polymerase dsDNA complexes.

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5) The structure-suggested roles of two residues (Q992 and Y999) are probed using biochemical studies. Given that the determined structures resemble others from DNA polymerase systems, a more detailed comparison would provide greater context and validation of the present work.

[Authors: We thank the reviewer for these suggestions. In the revised manuscript we incorporated multiple references to the relevant substrate complexes and cited previous work on T7, Klenow, TAQ, and PolG DNAPs.](#)

Reviewer #2 (Remarks to the Author):

Mitochondria uses phage-type RNA polymerase (mtRNAP) for transcribing mtDNA. In this study, authors used human mtRNAP to investigate substrate NTP loading to the active site and associated conformational changes of mtRNAP, DNA template and NTP by determining two cryo-EM structures of mtRNAP elongation complex (EC) with incoming NTP, named substrate entry complex (PDB: 8U8U) and substrate insertion complex. What new in this structural work compared with previous works is capturing the substrate entry complex, which shows for the first time that NTP binds O-helix of finger domain but not form base pair with DNA template, suggesting a mechanism of substrate selection prior to base pairing with DNA template. Finding is very interesting and provide new insight into the substrate selection of mtRNAP and related enzymes.

However, unfortunately, qualities of cryo-EM density map of both complexes and the final models are not good enough to claim new findings. For examples, densities of finger domain and NTP are relatively poor and the final model including some important residues (Y999, M995, Y956 and NTP [no density covering sugar, extra density next to alpha phosphate] in 8U8U; no density connecting between phosphate and sugar of NTP, somehow RNA 3'-OH and alpha phosphate of NTP is connected, both MgA and MgB do not fit density in

8U8V). I think lack of high-quality density around finger and NTP is due to mobility of finger and also particles belonging to each state were not separated well during cryo-EM data processing. Authors have to find better solution to separate particles and improve cryo-EM maps.

Response: We thank the reviewer for pointing out this deficiency. To enhance map quality, particles from the previous non-uniform refinement jobs were subjected to heterogeneous refinement (N = 3, hard classification). This process identified and excluded particles that couldn't be clearly classified as either "Entry Site" or "Insertion Site." These discarded particles resembled the EC but seemed distorted, likely due to orientation bias or motion blurring caused by the fingers domain opening and closing. The remaining particles underwent further refinement and sharpening, leading to significantly improved map quality (Figures RR1 and RR2).

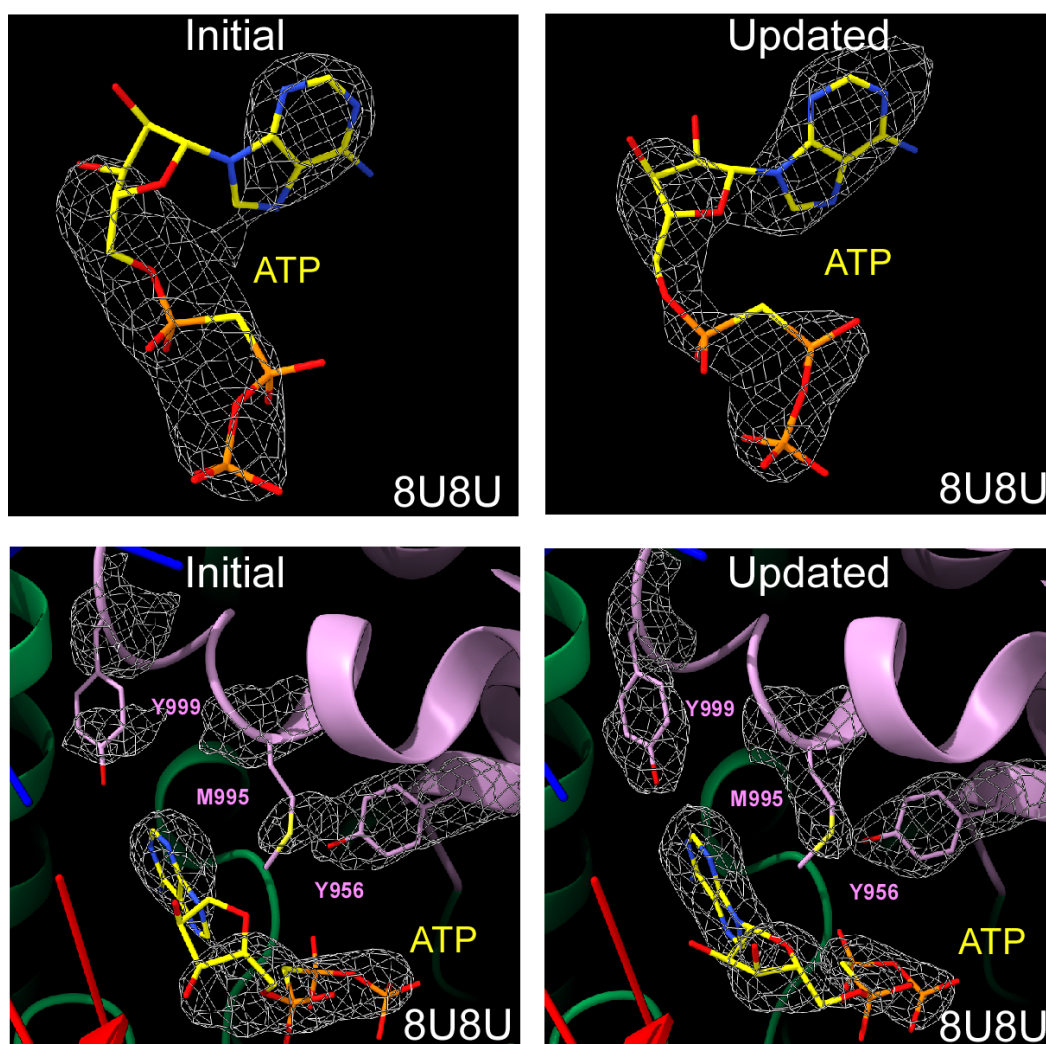


Figure RR1. Improvement of the cryo-EM density map quality for Structure I (Substrate Entry Site)

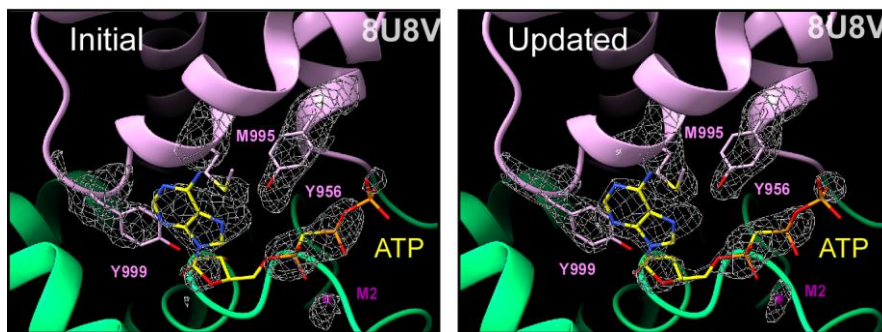
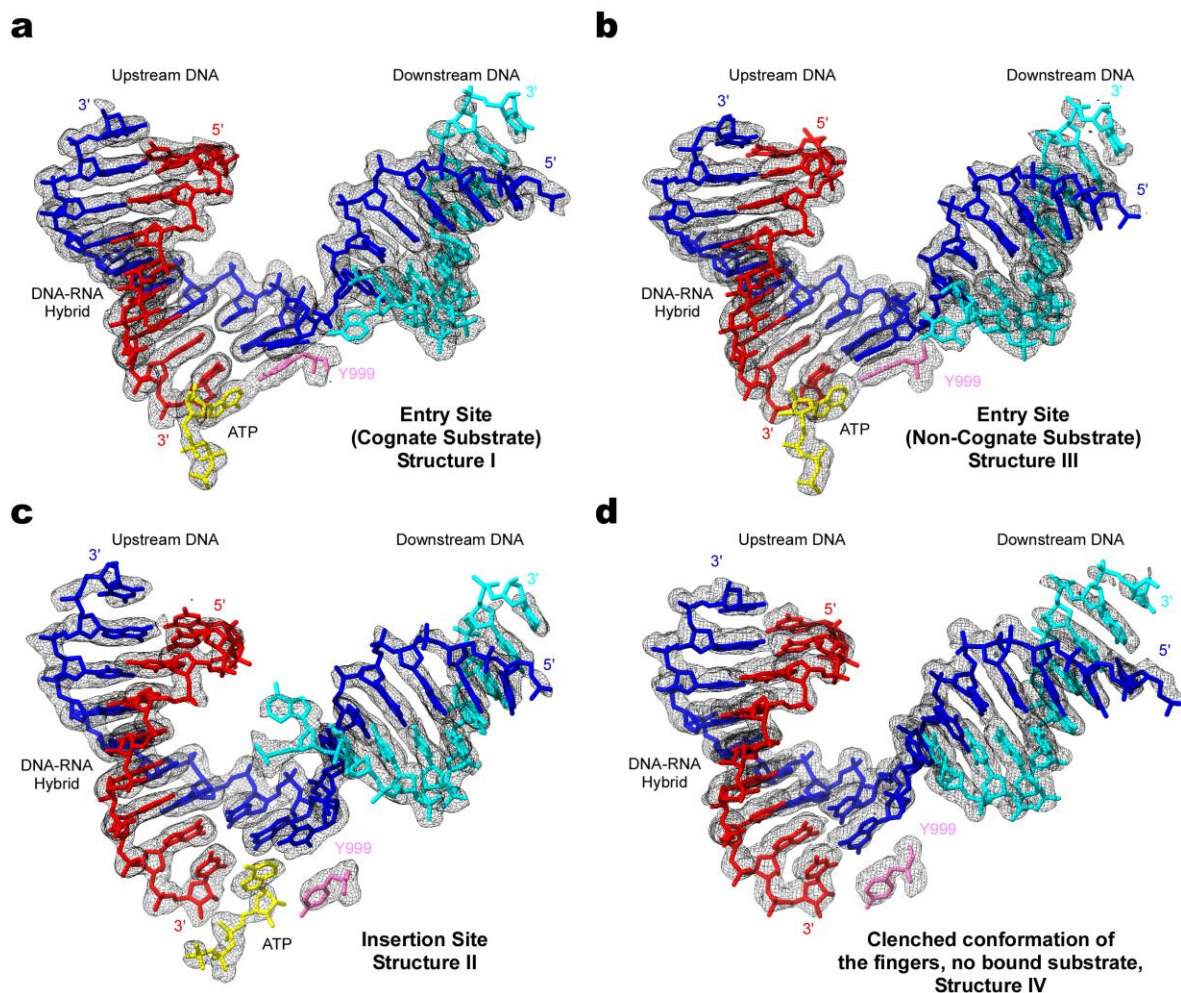


Figure RR2. Improvement of the Cryo-EM density map quality for Structure II (Substrate Insertion Site)

We also included new Figure S8 in Supplemental Figures, which attests to the quality of the CryoEM data for all structures.



One way is soaking non-cognate NTP to EC, which may form only substrate entry complex due to lack of base pairing with template DNA. Alternatively, increase concentration of AMPCPP (from 1 to 10 mM) to convert all EC to substrate insertion complex.

Response: We thank the reviewer for these thoughtful suggestions. While we did not observe any improvement of cryo-EM maps using AMPCPP titration, the experiment with the non-cognate NTP resulted in important data (Complex TWO), which is now included in the manuscript.

In short, we modified the sequence of the acceptor *n* base of the template strand of DNA, which converted the incoming AMPCPP into a non-cognate substrate. As predicted, the non-cognate substrate also binds to RNA polymerase in the Entry Site but not in the Insertion Site. These data (new Structure III) strengthen our conclusions about the mechanism of substrate binding and selection and are now discussed in the manuscript. We introduced new panels (Fig 1, Panel e, Fig 2, panel b) and provided data of cryo-EM analysis performed with EC with the bound non-cognate substrate (Supplemental Figures S1-8).

Importantly, a significant number of particles in Complex TWO represented yet another conformation of the EC - a complex lacking substrate ATP but having the fingers domain in a super-closed or “clenched” conformation (Structure IV, Fig 1e). This complex was not observed when the cognate

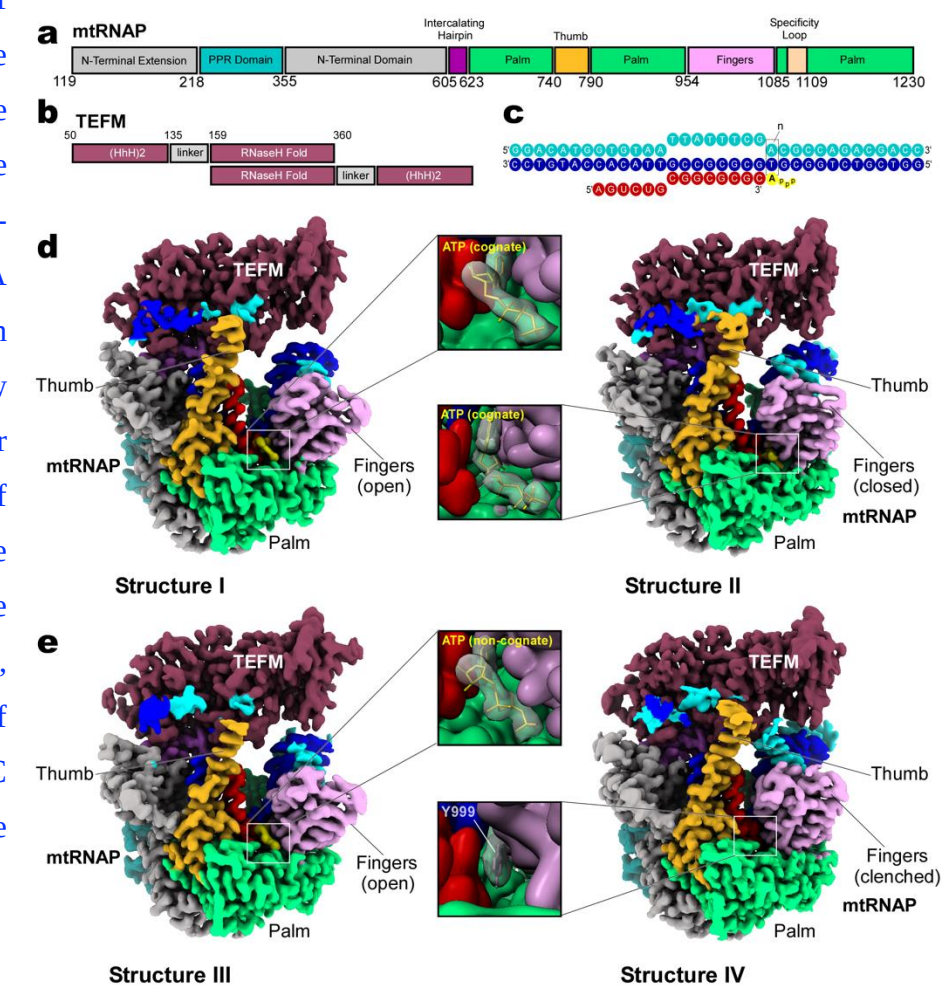


Fig 1 | Structures of human mitochondrial transcription elongation complexes with bound substrate
a,b, Schematic representation of the functional domains in mtRNAP and TEFM. The color code is used throughout.
c, Scaffold design used to assemble EC (Complex ONE). The *n* base pair (shown in the open box) was inverted in Complex TWO. NT DNA strand is shown in cyan, TS – in blue, and RNA – in red. The ATP analog is depicted as a yellow circle.
d, Cryo-EM densities of substrate-bound ECs from Complex ONE in two different conformational states. Close-up views of the active sites reveal cryo-EM density of the bound substrate ATP (yellow sticks).
e, Cryo-EM densities of ECs from Complex TWO in two different conformational states. Close-up views of the active sites reveal cryo-EM density of the bound non-cognate substrate ATP (Structure III) and Y999 residue (Structure IV).

substrate was present (Complex ONE) and could represent a conformation of RNAP after the incorrect nucleotide was rejected from the active site.

The new conformation of mtRNAP results from an additional rotation of the fingers domain by 13° and translation of the O/Y helices by $4\text{\AA}$ towards the template strand of DNA, as compared to the Insertion Site observed in Structure II (**Fig. 2e,f**). This movement appears to push the template strand of DNA away from the active site, with the sharp 90° bending of the DNA induced between bases $n-1$ and n , unlike the bend between bases n and $n+1$ in Structure II (**Fig. 2b,e,f**). This additional movement of the fingers domain results in rotating the n base out of the active site and brings the Y999 residue into the position normally occupied by substrate NTP in the Insertion Site, as evidenced by superimposing Structures IV and II using conserved residues in the palm subdomain (**Supplementary Video 4**). It is tempting to speculate that Substrate NTP bound in the Entry Site and subsequently moved into the Insertion Site during fingers closing would be rejected from the EC by fingers clenching observed in Structure IV.

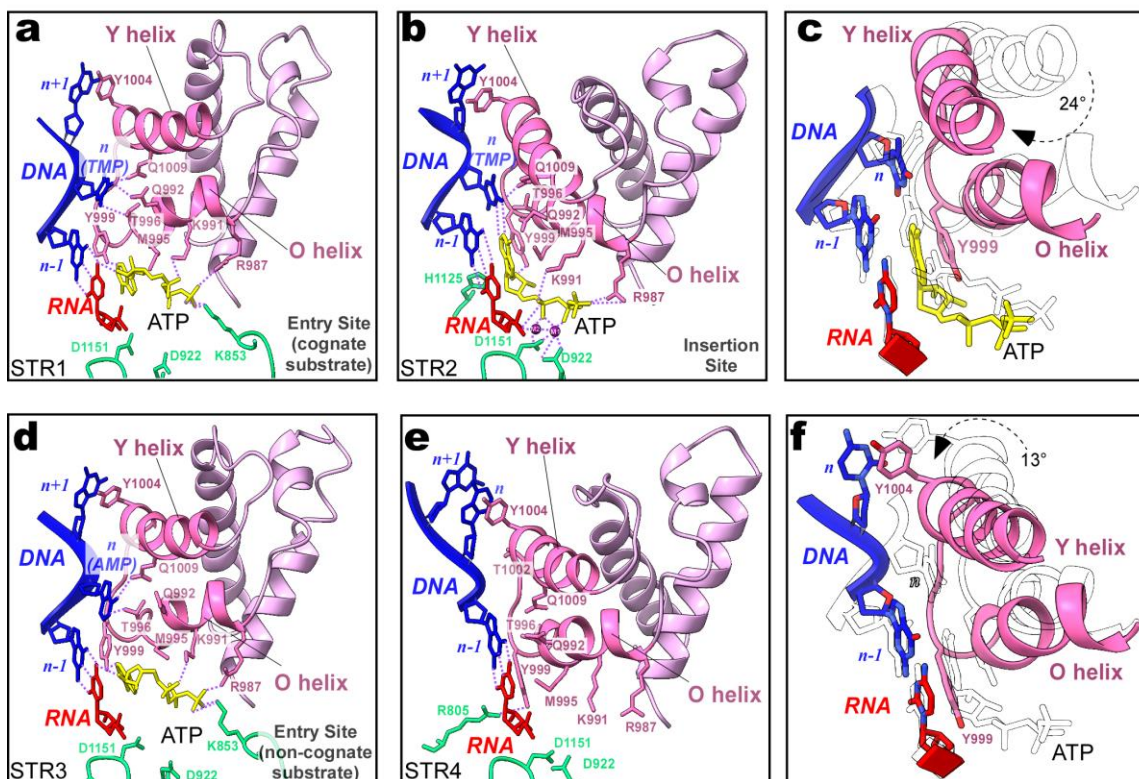


Fig 2 | Structural basis for substrate binding and selection by human mitochondrial RNAP

a,b, Close-up views of Substrate Entry and Substrate Insertion Sites of mtRNAP. The structures were aligned using the conserved residues in the palm domain. DNA, RNA, ATP, the palm domain, and the fingers domain are colored blue, red, yellow, green, and pink, respectively. Catalytic Mg^{2+} ions (M1 and M2) are shown as purple spheres; M1 was modeled based on homology modeling with T7 RNAP. Hydrogen bonds are shown as blue dashed lines.

c, Movement of the O and Y helices from Substrate Entry (white) and Substrate Insertion (colored) Sites.

d, Close-up view of the Substrate Entry Site with the bound non-cognate substrate

e, Close-up view of the Substrate Rejection Complex.

f, Movement of the O and Y helices from closed (white) to clenched (colored) conformation

We included our new structural data (Figure 2, d-e, Supplemental Figure 3-6) and updated the schematic depiction of NTP selection process (new Figure 4) to reflect these findings. New Supplemental Video 4 is introduced to illustrate the movement of the O/Y helices from closed to clenched conformation. The second to last paragraph of the manuscript was edited to reflect these findings:

“A two-pronged check-point mechanism of substrate selection by single-subunit RNA polymerases has been envisioned (**Fig. 4**)^{1,2}. The initial stage of substrate selection involves formation of the insipient base-pair with the acceptor DNA base, observed in the crystal structure of T7 RNAP EC² and likely represented by multiple intermediate complexes on a pathway from the Entry to Insertion Site in mtRNAP in Complex ONE (**Supplemental Figure S2**). The cognate substrate retained in the Pre-insertion Site travels into the Insertion Site where it is poised for a catalysis. When the non-cognate NTP is bound, imperfect base-pairing results in incomplete closing of the fingers domain and substrate dissociation from RNAP. This state of the fingers, termed the "ajar" conformation, has been observed in structural studies of DNA polymerase I bound to a non-cognate substrate²⁶. Because the ajar conformation remains relatively open, complete closure of the fingers domain is required to engage an induced fit mechanism of substrate selection at the Insertion Site^{27,28}. This mechanism relies on multiple local rearrangements of the active site residues, which assess the geometry of the base-pair^{29,30} and the conformation of the sugar moiety, thereby enabling discrimination against dNTPs³¹. These rearrangements likely trigger additional movement of the fingers domain to expel the non-cognate nucleotide, facilitated by the Y999 residue of the O helix, ultimately resulting in the formation of the Substrate Rejection Complex observed within Complex TWO dataset (**Fig. 2e,f**).

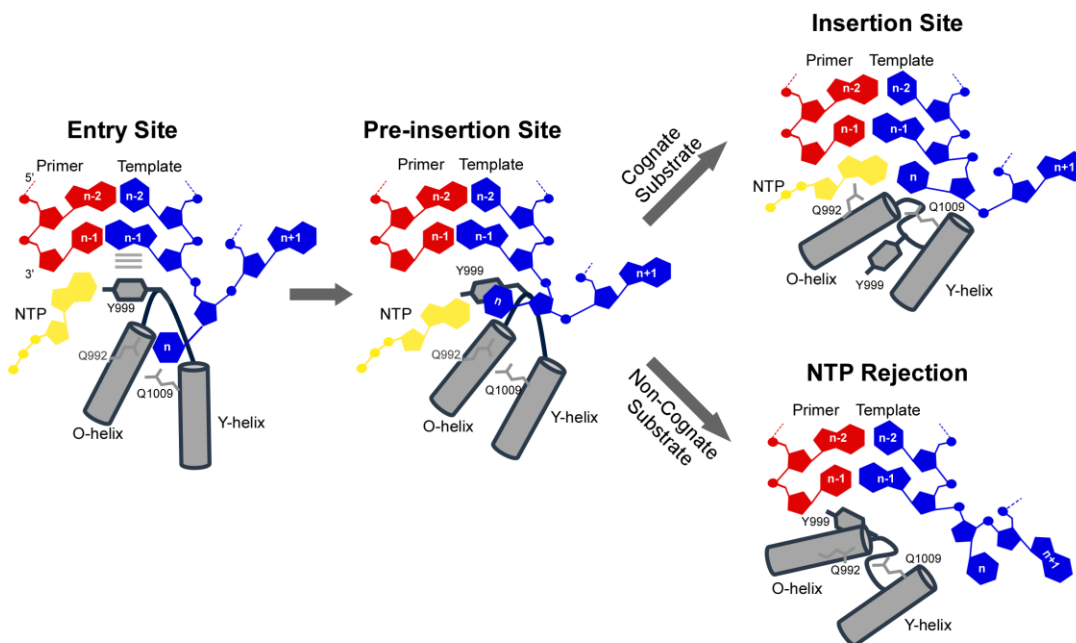


Fig 4 | The mechanism of substrate binding and selection in Pol A family of RNA and DNA polymerases

Schematic illustration of the major states along nucleotide addition pathway. Substrate binding in the Entry Site observed in RNAPs and DNAPs (8U8U, 9BDD, 4YFU); center top: substrate Pre-Insertion Site observed in RNAP (1S0V); top right - substrate Insertion Site (RNAPs and DNAPs: 8U8V; 1S76, 2HVI). Bottom right - NTP rejection complex (9BDC).

One of distinct features of the substrate entry complex compare with other elongation complex structures determined before is that the template DNA base at $i+1$ position (T) is inserted between O- and Y-helices. If authors aim to claim this is a general mechanism, they have to determine the structures of elongation complex containing different DNA bases at $i+1$ position (A, G, or C). DNA base at $i+1$ position interacts with Q1009, Q992 and T996 residues, thus functions of these residues have to be validated by site-directed mutagenesis.

Response: No base-specific interactions are expected between the DNA base n and polymerase, however, we followed the reviewer's suggestion and changed the base from purine (dTMP) to pyrimidine (dAMP) in Complex Two. We observed a nearly identical position of the n base in Structure III (Entry Site, non-cognate NTP, Fig 2 d) as compared to Structure I (Entry site, cognate NTP) and the same network of integrations with RNAP.

Per the reviewer's suggestion, we created variants of mtRNAP with alanine substitutions at residues Q992, T996, or Q1009. It was predicted that these variants would exhibit a defect in translocation, as mutations of similar residues in T7 RNAP (T636, PMID: 12460110 and Q649, PMID: 7929200) are known to cause deficiencies in enzyme processivity. Consistent with these expectations, our findings indicate that all three mtRNAP variants (Q992A, T996A, and Q1009A) displayed defects in the primer extension assay (illustrated in the new Figure 3, Panel c). Notably, the Q1009A mtRNAP variant showed the most significant effect, only able to extend the RNA primer by a single nucleotide. We have incorporated these new findings into the manuscript for discussion.

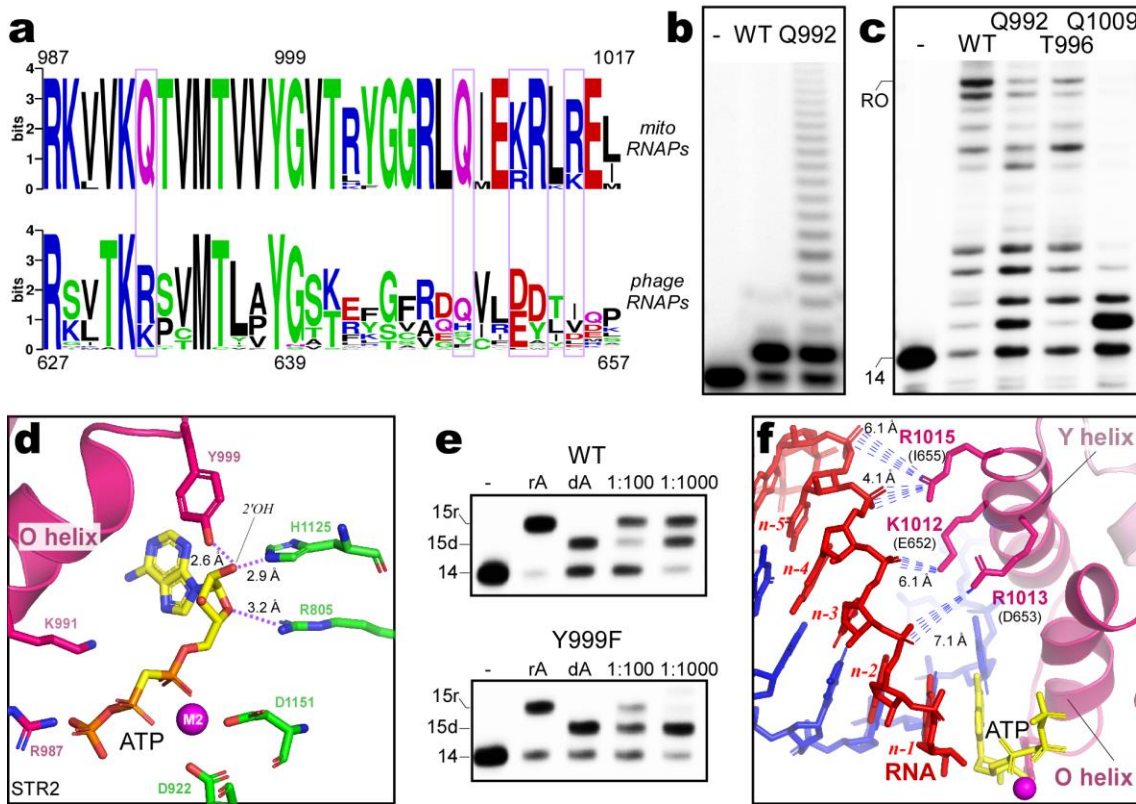


Fig 3 | Properties of human mitochondrial RNAP variants defective in translocation and rNTP recognition

a, Sequence logos of O/Y helices in mitochondrial (top) and bacteriophage (bottom) RNAPs. The residues that lack conservation between mitochondrial and phage RNAPs and are implicated in the NAC are shown in purple boxes.

b, Transcript slippage activity of the Q992A variant of mtRNAP

c, Primer extension assay performed with mtRNAP variants Q992A, T996A, and Q1009A

d, A close-up view of ribose moiety of NTP binding and recognition.

e, rNTP/dNTP competition assay. The RNA primer (14 nt) was extended in the presence of rNTP/dNTP mixtures, as indicated. The reaction products differ in mobility due to rAMP (15r) or dAMP (15d) incorporation.

f, Interactions between the Y helix and RNA in the Insertion Site of mtRNAP EC. Long-range electrostatic interactions are shown as dotted blue lines.

Reviewer #3 (Remarks to the Author):

In this manuscript, authors reported two cryoEM structures of human mitochondrial transcription elongation complexes that reveal substrate ATP bound in Entry and Insertion Sites, respectively. The entry site was not reported in any single-subunit RNAPs, in which the phosphate moiety is recognized by RNAP, the nucleobase moiety is not. This is different from previous reported pre-insertion state of T7 RNAP. In the insertion state, the substrate insertion state, the substrate base establishes Watson-Crick base pair the template base at the active site, the fingers domain is in closed state. Based on two new CryoEM structural data and previous reported pre-insertion state of T7 RNAP, authors propose a model for how human mitochondrial RNA polymerase recognize and incorporation substrate during transcription elongation. This work provides important mechanistic insights into human mitochondrial transcription.

The manuscript is well-written. The manuscript will be of general interest to the transcription field and broad readership of Nature Communications. One minor comment is listed here.

Response: We thank the reviewer for the kind words.

Could authors comment what factors make the new entry state is more stable for human mitochondrial transcription elongation complex, therefore authors are able to capture in this study, whereas no such state is captured for T7 RNAP or other single-subunit RNAP?

Response: We attribute the capture of new states to the cryo-EM technique employed. The most stabilized conformation appears to depict the initial substrate binding and the compact closed form of the fingers domain. However, other transient dynamic conformations observed in Complex ONE (Clusters 3-5) may become resolvable in the future. While these same conformations exist in crystallization drops, typically only one conformation is trapped in the crystal lattice. Furthermore, our findings indicate that the 3' OH group on the RNA (or DNA) primer plays a crucial role in substrate binding at the Entry site. This may explain why complexes formed using chain-terminating nucleotides, which lack a 3' OH group, do not include substrate NTP bound in the Entry Site.

Summary of other the major changes to the manuscript.

1. The paragraph in the Result Section describing an additional interaction of TEFM with DNA has been removed from the manuscript. TEFM-mtRNAP interaction is a minor point, which is not directly related to the main theme of the study. The corresponding Supplemental figure (S4) is removed from the manuscript.
2. DNA base nomenclature is changed from the "i" convention (used mostly in bacterial and eukaryotic transcription field) to "n", to make the study more relevant to the DNA polymerase field and to enable a direct comparison with RNA and DNA polymerases of Pol A family.
3. Figure 2 is divided into two figures (2 and 3) to accommodate new structural and biochemical data.
4. Additional Supplemental Figures were introduced: Fig S1, S3-6, cryo-EM data for new Complex TWO; Fig S7, Quality of the cryo-EM data for all structures. New Supplementary Table S2 reflects cryo-EM data for two new structures, III and IV.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed my critique.

Reviewer #2 (Remarks to the Author):

Authors improved cryo-EM map quality to claim their finding. I recommend to publish this work.

Reviewer #3 (Remarks to the Author):

This revision has addressed all my concerns. I recommend for publication.